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**The Contribution of Wild Mammals to the
Epidemiology of Tuberculosis (*Mycobacterium bovis*)
in New Zealand**

**A thesis presented
in partial fulfilment of the requirements for the degree of
Doctor of Philosophy
at
Massey University**

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AMENDMENTS

Page	Paragraph	Line	Comment
iii	1	4	insert <i>study</i> after Castlepoint longitudinal
10	4	5	change <i>hypneumoniae</i> to <i>hyopneumoniae</i>
13	1	2	replace <i>suggests</i> with <i>suggest</i>
13	2	4	replace <i>varies</i> with <i>vary</i>
14	2	6	insert <i>oral</i> before <i>inoculation</i>
14	3	7	omit <i>that</i> following <i>reported</i>
15	1	1	replace <i>patch</i> with <i>patches</i>
15	1	3	insert <i>M. bovis</i> after <i>where</i>
20	2	10	omit the second <i>that</i>
21	2	4	replace <i>size range from</i> with <i>diameter range of</i>
42	1	3	insert <i>of</i> after <i>favour</i>
44	2	1	omit first <i>have</i>
46	2	6	omit <i>that</i>
69	2	7	omit <i>in</i>
74	1	3	replace second <i>as</i> by <i>than</i>
77	3	1	insert <i>the</i> after <i>in</i>
83	2	last	replace <i>yet</i> by <i>while</i>
101	3	last	delete <i>that</i>
102	1	5	replace <i>curious</i> with <i>inquisitive</i>
112	3	2	omit <i>are</i> after <i>always</i>
113	3	8	replace <i>enlargement</i> with <i>enlargements</i>
119	2	1	insert <i>Prior to this study (Chapter 7) the hedgehog had</i> at the start of sentence
121	3	5	replace <i>reports</i> with <i>instances</i>
126	2	3	insert <i>locations</i> after <i>several</i>
127	1	2	replace <i>mycobacteria</i> with <i>mycobacterial diseases</i>
139	1	8	replace <i>have</i> with <i>had</i>
158	2	5	insert <i>grossly</i> after <i>have</i>
178	1	1	insert <i>own offspring</i> , after <i>to</i>
185	1	13	insert <i>LTA positive</i> after <i>five</i>
212	2	5	replace <i>mature</i> with <i>older</i>
221			the tonsillar lesions depicted in Figures 6-1 and 6-2 represent normal crypt pathology found in both cattle and deer. Infection with <i>M. bovis</i> may be the cause of such lesions in some animals, but this cannot be determined grossly
301	3	2	detailed necropsies, consisting of post mortem examination, followed by isolation of <i>M. bovis</i> and/or the finding acid-fast organisms during histopathological examination, were considered to be the 'gold standard' by which the other diagnostic methods were evaluated
346	1	6	insert <i>subsequently</i> before <i>died</i>
352	5	4	replace <i>this</i> with <i>these</i>
352	5	6	replace <i>This data was</i> with <i>These data were</i>
408	1	2	insert <i>within a radius of</i> after <i>within</i>
414	3	11	replace <i>A latter two</i> with <i>Two later</i>
425	2	1	replace <i>challenges</i> with <i>challenge</i>
469	10	1	insert <i>in feral pigs</i> after <i>Mycobacterium bovis</i>

Note: *Infected* is used in the text to refer to an animal or tissue which contains viable pathogenic micro-organisms, whereas *diseased*, is used to denote a situation where organs or tissues have pathologic changes as a result of such infection. The adjective '*tuberculous*' specifically refers to pathologic conditions caused by tubercle bacilli.

“To question all things - never to turn away from any difficulties, to accept no doctrine either from ourselves or from other people without rigid scrutiny by negative criticism; letting no fallacy, or incoherence, or confusion of thought, step by unperceived; above all to insist upon having the meaning of a word clearly understood before using it, and the meaning of a proposition before assenting to it, these are the lessons we learn from the ancient dialecticians.” - John Stuart Mill

Abstract

The objective of these studies was twofold. The primary aim was to gain a better understanding of the role of free-living mammalian species, other than possums, in the epidemiology of wildlife tuberculosis in New Zealand. The other objective was to continue the operation of the Castlepoint longitudinal so that hypotheses regarding the epidemiology of *M. bovis* infection in possums could be further refined and clarified.

Of the wild carnivores found in New Zealand, the disease persists at high prevalence only in ferrets, and is probably maintained principally by ingestion of tuberculous carrion. Although a moderate number of ferrets excrete *M. bovis* orally, there appears to be only minor intraspecific transmission by bite wounding. Although cats and stoats can also become infected through scavenging, they appear to be less susceptible to oral infection than ferrets. There is no substantial evidence to suggest that any of New Zealand's free-living carnivores are likely to be reservoir hosts of *M. bovis*.

Observational studies involving twelve domestic red deer suggested that cervids probably become infected through close inspection and investigation of moribund tuberculous possums, and that the likelihood of exposure to *M. bovis* was related to the curiosity and social ranking of the deer. Necropsies conducted principally on wild red deer and involving 152 animals provided evidence to suggest that significant bacillary excretion from infected deer was uncommon, and that only the few with advanced disease had the potential to be highly infectious. However, behavioural phenomena and disease characteristics preclude the ready transmission of disease amongst cohorts. There is now strong evidence to suggest that a high prevalence of tuberculosis infection in wild deer can only be maintained through contact with infected possums. However, deer may still be able to maintain the disease amongst themselves, albeit at a low prevalence, in the absence of infection in possums. This study also confirmed the importance of lymphoepithelial tissues, such as the oropharyngeal and nasopharyngeal tonsils, as primary sites for the establishment of *M. bovis* infection, and the subsequent excretion of organisms in deer.

The gross and histopathological appearance of the lesions found in six infected hedgehogs are described. It is likely that infection arose from the scavenging

behaviour of hedgehogs. The moderate prevalence (3.9%) of tuberculosis in these animals, combined with their small home ranges may allow them to be used successfully in wildlife surveys to pinpoint the locality in which tuberculous possums have died.

To gain an understanding of the potential role of wild pigs, goats, sheep, rabbits, hares, rats and mice in the dynamics of *Mycobacterium bovis* infection in free-ranging animals, numbers of these species were examined for evidence of infection. Of these, only the pig appears to have sufficient potential for intraspecific transmission to be of concern in tuberculosis control programmes. Sheep and goats appear to be simply spillover hosts, which may have a limited role in disease amplification following possible, but limited, intraspecific transmission. Rodents and lagomorphs are most unlikely to play any substantial role in the epidemiology of tuberculosis in New Zealand, under current circumstances.

A longitudinal study was established in 1989 to examine the disease behaviour in an infected possum population on a farm in the southern North Island of New Zealand, by trapping, using a fixed set of 295 traps for at least 3 days per month. Animals captured were examined at 2 monthly intervals for evidence of tuberculosis. During the first 5.5 years of this project over 900 individual possums were captured and tagged. Blood was collected from each possum examined, and the sera retained were stored frozen. Using these stored sera, three indirect ELISAs were evaluated as diagnostic tests for tuberculosis in possums. All ELISAs had low sensitivity when a cutoff selected to maximise the specificity was chosen. None of the ELISAs reliably detected possums infected with tuberculosis and they therefore have limited value for epidemiological studies. The lymphocyte transformation assays performed on blood taken from possums was estimated to have a sensitivity for detection of tuberculosis of approximately 80%, when the specificity was set at 99%. The lymphocyte transformation assay was the best of the *in vivo* tests evaluated, with the moderate sensitivity allowing it to be used with a degree of confidence to retrospectively diagnose disease, and aid the development of hypotheses regarding the epidemiology of tuberculosis in possums. The evaluated tests were applied retrospectively to sera and blood samples from possums from the Castlepoint longitudinal study. The additional data arising from these assays suggested that perhaps as few as one fifth of study site possums which had contact with *M. bovis*

had been previously detected as infected by clinical examination. A proportion of these test positive/examination negative animals may have been exhibiting resistance to *M. bovis* infection, and/or had resolved lesions or cryptic infection. Such animals may have formed a pool of possums in which future reactivation of tuberculosis was possible. The time from earliest evidence of infection till death, in those possums which showed clinical disease, varied from months to several years.

Cortisol assays performed on stored sera, and monitoring of trends in body weight, were used to investigate the role of stressful environmental phenomena in the epidemiology of tuberculosis in possums. Major stressful periods involving inadequate nutrition, heat, cold and moisture stress appear to precipitate severe tuberculosis outbreaks, which are believed to have their origins in the reactivation of subclinical/latent infection in the population. As the period of pre-clinical disease varies substantially, and can be as long as several years, this epidemic of tuberculosis takes several years to subside. Thereafter a small number of clinically diseased possums are likely to be restricted to “hot spots” conducive to transmission of *M. bovis*.

Isolates of *M. bovis* recovered from a variety of species, both wild and domestic, in the Castlepoint environs, and in particular the Castlepoint study site, were subjected to restriction endonuclease analysis to DNA fingerprint the strains present, and hence gain a better understanding of the inter- and intraspecific epidemiology of tuberculosis. The results do not challenge the accepted view of possums being the major reservoir hosts of tuberculosis in the Wairarapa. There was also no evidence to suggest that host adaptation of *M. bovis* has occurred, except in the case of possums, where they appear to be able to maintain clusters of individuals infected with particular restriction types, in microhabitats for at least 5 year periods. The occurrence of newly introduced restriction types has made possible new observations on the epidemiology of infection, including the documentation of the occurrence of latent infections, duration of primary progressive disease in newly infected possums (7-8 months), and the likely occurrence of post-primary reactivation of tuberculosis.

Acknowledgments

I was indeed fortunate to be offered, and able to take up the position of research officer in Professor Roger Morris's epidemiology group, as this step catapulted me headlong into a tough, but worthwhile struggle to enlarge my professional horizons, expand my interests in wildlife, and I hope, to make a significant contribution to solving the problem of bovine tuberculosis in New Zealand. I must first thank NSW Agriculture, for allowing me the opportunity to undertake these studies. To Roger Morris I am also truly grateful for this rare chance to conduct PhD studies under such fortunate circumstances. Under Roger's tutelage I have learned a great deal, and am wiser for the experience. To him I owe my thanks.

To Dirk Pfeiffer, my friend, mentor and co-supervisor, I am deeply indebted. He ably assisted the transition from raw novice with figures to one now competent to take on many statistical challenges. Dirk was often brimming with great ideas, enthusiasm and good cheer, when these were at low ebb in myself. He was prepared to listen to ideas in an impartial manner, despite them often being contrary to the "accepted wisdom" on tuberculosis. I miss his company and the daily debriefing chats we had on our bicycle rides home from the university.

My other supervisor, Associate Professor Peter Wilson, was instrumental in teaching me something of the husbandry and diseases of deer, topics of which I possessed complete ignorance before enrolling in the PhD studies. I thank Peter for his tuition, encouragement, friendship and prompt attention to my needs throughout my stay at Massey university.

To my colleagues, particularly Carola Sauter, and support staff, and especially those involved in the study of tuberculosis, I am indebted for their help and company on many occasions. There were many who contributed substantially in various ways, and these folk are acknowledged at the end of each chapter.

The management of the Castlepoint study site presented its share of difficulties, none insurmountable, but challenging none the less. Without the friendly co-operation of Ron Goile, manager of Waio station, and his partner, Donna Lewis, these problems may have been difficult to overcome. Their help was sincerely appreciated, as was their outstanding contribution to the longitudinal study.

To my family I am deeply indebted and sincerely appreciative of their support. They were separated from their father and husband for the first six months of the studies, dragged unwillingly across the Tasman to settle in a foreign urban environment and then uprooted again to return to Australia. Whilst in New Zealand they endured my prolonged absences, disappearances on weekends and late nights working on the thesis, with little complaint. I sincerely apologise for the neglect of family matters, and the time for which the PhD studies precluded me spending with my loved ones. I hope in the fullness of time that the completion of the studies will bring rewards which have a tangible benefit to my family.



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Table of Abbreviations and Units

Codes	Descriptions
%	Percentage
°C	Degrees celsius
µg	Microgram
µm	Micrometre
ACTH	Adrenocorticotrophic hormone
AFB	Acid-fast bacillus(i)
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AVP	Arginine vasopressin
BALT	Bronchus-associated lymphoid tissue
B/A SI	Bovine to avian stimulation index
BCG	Bacille Calmette-Guérin
bcg ^r	Bacille Calmette-Guérin resistant genotype
bcg ^s	Bacille Calmette-Guérin susceptible genotype
BUN	Blood urea nitrogen
CALT	Conjunctiva-associated lymphoid tissue
CBG	Cortisol binding globulin
cfu	Colony forming unit
CI	Condition index
cm	Centimetre
CMI	Cell-mediated immunity
Con A	Concanavalin A
cpm	Counts per minute
CRH	Corticotrophin releasing hormone
CV	Coefficient of variation
Delta Con A	Concanavalin A stimulated, minus control counts in lymphocyte transformation assays
DF	Degrees of freedom
dl	Decilitre
DNA	Deoxyribonucleic acid
DR	Direct repeat elements
DSP	Deer slaughter premises
DTH	Delayed-type hypersensitivity
ELISA	Enzyme-linked immunosorbent assay
Eta squared	Approximation for the amount of variance explained by a term in a general linear model
F - value	Value of the F statistic in a general linear model
g	Gram
GALT	Gut-associated lymphoid tissue
GC	Glucocorticoid
GPH	Game packing house
H&E	Haematoxylin and Eosin stain
ha	Hectare
Hg	Mercury
HPA	Hypothalamo-pituitary-adrenal
HPF	High power microscope field
IFN-γ	Interferon gamma
Ig	Immunoglobulin

Table of abbreviations continued

Codes	Descriptions
IL	Interleukin
kg	Kilogram
km ²	Square kilometre
LH	Luteinising hormone
ln.	Lymph node
lnn.	Lymph nodes
LPF	Low power microscope field
LTA	Lymphocyte transformation assay
MAF	Ministry of Agriculture and Fisheries
MALT	Mucosa-associated lymphoid tissue
Max	Maximum
mg	Milligram
MHC	Major histocompatibility complex
Min	Minimum
mm	Millimetre
NK cell	Natural killer cell
ng	nanogram
NGL	No gross lesions
OR	Odds ratio
P or p	Probability
PAM	Pulmonary alveolar macrophage
PCR	Polymerase chain reaction
PGRS	Polymorphic GC-rich repetitive sequence
PHA	Phytohaemagglutinin
PIM	Pulmonary intravascular macrophage
pO ₂	Oxygen tension
POMC	Proopiomelanocortin
PPD	Purified protein derivative
PWM	Poke weed mitogen
R	Correlation coefficient
R ²	Coefficient of determination
REA	Restriction endonuclease analysis
RNA	Ribonucleic acid
RFLP	Restriction fragment length polymorphism
s	second
SD	Standard deviation
SE	Standard error
SI	Stimulation index
SRBC	Sheep red blood cells
T ₃	Triiodothyronine
T ₄	Thyroxine
Tb	Tuberculosis
TbL	Tuberculous lesion
Th1	T - helper 1
Th2	T - helper 2
TNF	Tumour necrosis factor
ZN	Ziehl-Neelsen stain
95%CI	95% Confidence interval

CHAPTER 1

INTRODUCTION

In 1989, as part of a national integrated effort, a series of epidemiological studies began under the direction of Professor Roger Morris at Massey University, Palmerston North. These were undertaken to clarify the role of the brushtail possum in the tuberculosis problem of New Zealand livestock. These investigations aimed to reduce the incidence of infection in cattle and deer herds through aiding the design and execution of sound disease control programmes. At that time it was clear that the currently used possum destruction methods were inadequate to eliminate the disease from possums. From the examination of the situation, it was concluded that better definition of the epidemiology of tuberculosis in possums and other mammals was necessary to clarify the roles of the known and potential hosts of tuberculosis in New Zealand. Using sound epidemiological methods, it was thought possible to adopt a structured systems approach, whereby identification and implementation of effective control of tuberculosis in domestic cattle and deer populations exposed to a variety of infected wild species, could be instigated. This study forms the third major stage of a long-term research effort to achieve these goals.

The studies described within this thesis have developed from the longitudinal study at Castlepoint, commenced by Pfeiffer (1994), and subsequently carried on by Jackson (1995). Parts of this thesis are based upon data arising from this study and its continuation. Other sections are devoted to the exploration of the role of tuberculous hosts other than possums, in particular deer and ferrets, as the part played by these and other wild infected hosts such as, cats and pigs was uncertain, and regularly questioned by farmers, pest managers and scientists. Both deer and ferrets were believed by some scientists to have a significant role in the epidemiology of tuberculosis, both being implicated as maintenance hosts, deer in transmitting infection to uninfected possum populations, and ferrets in passing the infection to domestic stock.

The study of the other wild hosts of *Mycobacterium bovis* was initially focused upon the Castlepoint area, as there was already a substantial investment in, and body of data relating to the infection in possums, including the restriction endonuclease analysis (REA) types of *M. bovis* involved. However, due to the low density of alternative wild hosts, and other practical limitations of the study site and

environs, the sites of material collection and animals for necropsy was broadened as collaborative opportunities arose.

Within the veterinary scientific community, a sense of complacency regarding infection with *M. bovis* arose during the last 40 years, as many developed countries made good progress towards eradication of bovine tuberculosis. However, the discovery of maintenance wildlife hosts such as possums and badgers, has spurred new efforts to better understand the pathogenesis and epidemiology of the disease, particularly in these free-living hosts. Unfortunately, the study of tuberculosis in these species has been hampered by the inherent difficulties involved in investigating a disease in free-ranging, non-domesticated and nocturnal hosts. Where possible, in this thesis, quantitative epidemiological techniques have been employed to ensure that inferences and conclusions arising from the data were soundly based. However, as with much research, many conclusions are derived by inference from multiple sources of information, and in this thesis some of these sources have come from the study of mycobacterioses in other species, as it was believed that broader approach to the literature (especially regarding pathogenesis) was necessary to escape from the “restrictive” dogma surrounding *M. bovis* infection which has arisen over the past 100 years of research into these fascinating organisms, and the disease which they cause.

CHAPTER 2

REVIEW OF THE LITERATURE

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Aspects of disease pathogenesis

The following review attempts to explain some facets of the pathogenesis of tuberculosis in mammals, so that with a better understanding of the mode of infection, bacillary dissemination within the body, and the immunological responses, the reader is better prepared follow the arguments presented in the succeeding chapters. Some hypotheses presented are at variance to the conventionally accepted wisdom on bovine tuberculosis, which has arisen from over 100 years of detailed and dedicated research. However, I found this review, the development of new hypotheses and the re-working of older forgotten concepts on the disease to be a necessary task, required to explain observations which had been made, and for which the accepted ideas on pathogenesis were unhelpful. To do this, much old literature has been revisited and re-presented, in conjunction with more recent research, in a form which I hope does justice to those who have worked before me in helping to unravel the secrets of this most persistent and successful obligate mammalian pathogen - *Mycobacterium bovis*.

Portals of mycobacterial entry into the body

Mucosa-associated lymphoid tissues (MALT)

These tissues are composed of a ring of lymphoid structures circling the oropharynx (Waldeyer's ring), principally the oropharyngeal and nasopharyngeal tonsils but also including tubal and lingual tonsils in some species, intestinal Peyer's patches, aggregated lymphoid nodules in the large intestine, isolated lymphoid nodules throughout the gut from the oesophagus to the anus, the bronchus-associated lymphoid tissue (BALT) and conjunctiva-associated lymphoid tissue (CALT) (Carlson and Owen, 1987; Fix and Arp, 1991a; Liebler *et al.*, 1991). These components of the immune system are characterised by a specialised lymphoepithelium containing M-cells which take up macromolecules and micro-organisms; the associated underlying secondary lymphoid follicles located in the lamina propria; and by the absence of afferent lymphatics. Although there are species and site differences in gross anatomical detail, especially in the oropharyngeal tonsils, the fine structure and functional characteristics appear to be similar across species, whether eutherian or marsupial (Hemsley, *et al.*, 1996b).

One of the main functions of the lymphoepithelium is to provide a favourable environment for the contact between antigens, intraepithelial lymphocytes and

antigen-presenting cells (Perry, 1994). MALT has the complex task of both recognising pathogens and mounting an appropriate immune response, and recognising antigens which are non-pathogenic, and preventing inappropriate tissue damaging or energy wasting responses (Carlson and Owen, 1987). The lymphoid cells of the follicle domes limit the systemic dissemination of antigens and bacteria, whilst simultaneously initiating immune responses, particularly those aimed to enhance mucosal defences (Owen, 1991). There is some evidence arising from experiments involving oral immunisation with *Candida albicans*, to suggest that the response of MALT immunocytes is likely to be of a T-helper 2 (Th2) type (humoral) response, whereas concurrent systemic responses are of a T-helper 1 (Th1) type, involving cell mediated immunity (CMI) (Jensen *et al.*, 1996). Infectious agents which are not trapped by the follicular macrophages after transport through the epithelium enter efferent lymphatics and may disseminate to distant sites. The mucosal immune system thus has the ability to recognise different groups of antigens, and has evolved a battery of responses from which an appropriate reaction can be orchestrated. Its function is primarily involved in keeping antigens out, initiating immune responses, inducing immune cells that relocate to distant effector sites, and preventing the interaction of harmful substances with the epithelium. However, functionally MALT also provides gaps in the integrity of mucosal barriers whereby pathogens may gain entry to the host.

The surface of lymphoepithelial tissues is not uniform and contains patches of normal epithelium for that site (squamous or villous), interspersed with areas of reticulated epithelium which is composed of M-cells, lymphoid cells and the normal epithelial cell types of the surrounding mucosa. The M-cells ('M' for microfold bearing or membranous) function as selective, but active macromolecule and micro-organism uptake and transport cells (Carlson and Owen, 1987). They cannot be identified by light microscopy, but when visualised with the aid of electron microscopy, they are seen to have an apical surface of microfolds and short irregular microvilli. Vesicles are abundant in the apical cytoplasm, and these are the means by which micro-organisms and macromolecules are endocytosed and transported through the M-cell (Wolf and Bye, 1984), and apparently discharged into the supranuclear intercellular spaces (Liebler *et al.*, 1995).

Reticulated lymphoepithelial patches are associated with disruptions of the basement membrane, desquamation of the upper epithelial layers and infiltration with small blood vessels, both capillaries and high endothelial venules (Perry, 1994). The epithelium in these areas is infiltrated with macrophages, T and B lymphocytes, plasma cells and occasional polymorphonuclear leucocytes and dendritic cells (Figure 2-1). These accessory cells have been seen to migrate through the fenestrated basement membrane of the lymphoepithelium and move through intraepithelial passageways, which form in response to the flexible nature of the M-cells and their attachments (Belz and Heath, 1995a). In this way, it is thought that the M-cells facilitate the ingress of lymphoid cells into the lumen of the viscus (Wolf and Bye, 1984; Belz and Heath, 1995a). These lymphoid cells which escape into the viscus or crypt lumens form part of the intraluminal cellular debris. The ability of the cytoskeleton to rearrange itself and redistribute desmosomal contacts indicates that this epithelium is dynamic and responsive, presumably to antigenic stimulation. Direct trans-epithelial access of antigens may stimulate greater influx of non-epithelial cells and cause a thinning of the epithelium. In the extreme, inflammatory reactions superimposed on top of the normal pattern of reticulation, will result in more lymphoid cells being expelled from the surface of the epithelium and will lead to loss of integrity of the epithelium altogether, with direct exposure of the underlying lymphoid cells to the lumen of the viscus (Payne and Derbyshire, 1963; Chen *et al.*, 1991).

The lymphoepithelial tissues contain both T and B lymphocytes. The B-cells are found principally in the follicles, where active proliferation takes place in the germinal centres, whereas the T-cells are located mainly in the domes overlying the follicles, the parafollicular areas and in the lymphoepithelium (Hemsley *et al.*, 1996b). There is a great volume of lymphocyte traffic into and out of these sites, with most of the ingress and egress via the high endothelial venules (Carlson and Owen, 1987; Brandtzaeg, 1988). B-cells are known to disperse principally to other parts of the common mucosal immune system where they are involved in the development of secretory Ig A responses (Wolf and Bye, 1984). The lymphocyte pool in MALT includes both T-helper and suppresser cells. The latter are thought to play a role in the induction of tolerance to some antigens, and may also suppress local inflammatory responses which would disturb the function of the MALT (Bienenstock, 1985). There is also a range of accessory cells which are especially

numerous in the subepithelial domes, and primarily include dendritic cells and a lesser number of macrophages, both of which are presumed to play an active role in phagocytosis and presentation of antigens to the adjacent lymphocytes (Williams and Rowland, 1972; Kelsall and Strober, 1996).

It is unknown if the M-cells have a role in antigen processing or presentation, but recently Allan *et al.* (1993) found that M-cells were able to express MHC class II antigen on their cytoplasmic membrane, and that the M-cell infiltrating lymphocytes were not located randomly, but were found principally in branches of supranuclear M-cell cytoplasm (Regoli *et al.*, 1995), thus strongly suggesting that they are capable of antigen processing.

Full development of lymphoepithelial tissues is germane to the acquisition of a competent mucosal immune system. Development varies between tissue site and species involved, but all develop prenatally, with maturation being regulated by antigenic stimulation and other unidentified factors (Chen *et al.*, 1990). Peyer's patches in sheep, for example, are fully functional at birth, whereas the lymphoepithelium is absent from BALT in adult horses, cattle and sheep, but will develop in response to pneumonia in the lungs of cattle and sheep (Anderson and Moore, 1986). The nasopharyngeal tonsils are particularly prominent and furrowed in immature ruminants, but atrophy with age to the extent that they are barely discernible in older animals (A. Davies, pers. comm.; Schuh and Oliphant, 1992).

There are a range of bacteria, viruses and protozoa, which are capable of adhering to, and being endocytosed by M-cells as part of the normal infectious process, whereby host tissue access is gained. *Mycobacterium* spp., *Listeria monocytogenes*, *Brucella abortus*, *Yersinia enterocolitica*, *Shigella flexneri*, *Salmonella typhimurium*, *Actinobacillus hypneumoniae*, *Vibrio cholerae*, *Chlamydia* spp. and human immunodeficiency virus, rotavirus, adenovirus and reovirus, have all been shown to enter host tissues by this means (Kumagai, 1922; Payne and Rankin 1961; Wolf *et al.*, 1981; Inman and Cantey, 1983; Fujimura, 1986; Ackerman *et al.*, 1988; Buller and Moxley, 1988; Momotani *et al.*, 1988; Amerongen *et al.*, 1991; Okamoto *et al.*, 1994; Autenrieth and Firsching, 1996). Larger antigens such as *Giardia muris*, *Eimeria coecicola* and cryptosporidia are also taken up by the M-cells and subsequently passed on to the subjacent macrophages (Owen *et al.*, 1981; Marcial and Madara, 1986; Pakandl *et al.*, 1996). *Pasteurella* spp. and *Actinobacillus*

pleuropneumoniae are also known to colonise the tonsils of calves and pigs respectively, presumably after uptake by the epithelium (Frank and Briggs, 1992; Møller *et al.*, 1993; Ackermann *et al.*, 1994). This process of endocytosis is selective, and does not facilitate the uptake of all bacteria. *Escherichia coli*, for example, have been shown to adhere to the apical microvilli, but are not endocytosed (Inman and Cantey, 1983).

Oropharyngeal Tonsils (palatine tonsils)

Some investigators have for a considerable period realised the importance of the tonsil in the acquisition of bacterial infection. Indeed as early as 1950, Wright (1950), in a review of the function of the human tonsil remarked that it was “a matter of common knowledge that tuberculous infection of the lymphatic glands occurs most frequently in the neck, and there is considerable weight of evidence to suggest that this infection gains entrance through the tonsil. Indeed, in some half of the cases of such infection, microscopic investigation will produce evidence of tuberculosis in the tonsil itself”. It was however unfortunate that no references, or supporting evidence were presented to substantiate Wright’s observations, and that similar studies have not been described since in the medical literature. The typical pathology of the bovine oropharyngeal tonsil has been described by Payne and Derbyshire (1963). Inflammatory reaction was a common feature, demonstrable by both focal and diffuse infiltrations with polymorphonuclear leucocytes and eosinophils, and development of small abscesses and granulomas in most tonsils. Similar changes were reported in the tonsils of cattle and buffalo by Pelagalli *et al.* (1982). Especially common were granulomas with giant cells containing vegetable foreign bodies. The epithelium could become so congested with lymphocytes, macrophages and polymorphonuclear leucocytes that its structure became ill-defined. Macrophages laden with bacilli were seen adjacent to the follicle lymphoid tissue. Payne and Derbyshire (1963) proposed that the tonsil was in an ideal site to sample the bacteria ingested with food. These bacteria were thought to invade the crypts, gain entry to the lymphatic tissues, with some escaping the tonsil by carriage in macrophages via efferent lymphatics, establishing infection in the draining medial retropharyngeal lymph nodes, which they showed to contain similar species of bacteria to those isolated from the tonsil.

In some species, e.g. the ruminants, a system of crypts increases the surface area of the lymphoepithelium (Nickel, *et al.*, 1979). Ruminants also have tonsils recessed in the pharyngeal wall to reduce the antigenic bombardment and physical irritation involved with often having food or regurgitated ingesta in the mouth. Muscular activity of the pharynx draws material into the crypts of ruminants during mastication. Carnivores however have simpler tonsils which protrude into the lumen of the pharynx thus ensuring exposure to ingested antigens during the brief period for which food is in the mouth. Lymphatic drainage from the tonsils passes to the medial retropharyngeal lymph nodes in most species, but the exact pathways have not been described in detail, except for the pig and dog (Belz and Heath, 1994; Belz and Heath 1995b). In pigs the drainage also passes to the mandibular lymph nodes and to the superficial cervical lymph nodes. It is possible that some of the tonsillar drainage may pass to the mandibular lymph nodes in other species as well.

Nasopharyngeal tonsil (pharyngeal tonsil, adenoid)

This organ, which lies in the roof of the nasopharynx, at the caudal end of the pharyngeal septum, is often overlooked, as grossly it frequently appears similar to the rest of the nasopharyngeal epithelium (Nickel *et al.*, 1979). In some species, such as sheep and cattle, the tissue is characterised by furrowing, especially in young animals, whereas in other species it is only recognisable grossly through the underlying follicles giving the surface a granular (domed) appearance. This organ was the only site in the respiratory system of sheep in which particulate antigens (carbon particles) were taken up for presentation to immunocytes lying in, or underneath the lymphoepithelium (Chen *et al.*, 1989). This carbon uptake was shown to occur within 30 minutes of antigen instillation. Although other parts of the nasal, tracheal and bronchial mucosa examined possessed aggregations of lymphoid tissue, none were overlain by lymphoepithelium, as was also the case in a study of the nasal mucosa of a variety of primate species by Loo and Chin, (1974). This lymphoepithelium contains M-cells similar to other MALT (Chen *et al.*, 1991; Schuh and Oliphant, 1992). It has been suggested that the nasopharyngeal tonsil is strategically situated, such that antigenic material trapped after inhalation into the nasal cavity, is preferentially swept by muco-ciliary action over the surface of the organ, prior to swallowing (Wright, 1961; Schuh and Oliphant, 1992). Lymphatic drainage is likely to flow directly to the medial retropharyngeal lymph node, which

lies nearby in most species. The advanced development in the foetus, strategic location, size, rugose surface and abundant lymphoid tissue suggests that the nasopharyngeal tonsil is the major (and probably the only) mucosa-associated lymphoid tissue of the nasal cavity (Schuh and Oliphant, 1992).

Peyer's patches

These are groups of lymphoid follicles which occur in the small intestine, distributed from the stomach to the ileocaecal junction. They are replaced by similar lymphoid aggregations with identical function in the large intestine. The number and size of the Peyer's patches varies between species and with the age of the animal. In the brushtail possum (*Trichosurus vulpecula*) for instance, there are approximately six to thirteen Peyer's patches scattered throughout the small intestine (Hemsley, *et al.*, 1996a), whereas in pigs there are 25 to 35 small but permanent patches, and one up to 2.5 m long in the ileum which does not persist beyond one year of age (Stokes *et al.*, 1994). Many gut immune cells are found outside the Peyer's patches, and these are not randomly distributed, but are compartmentalised, often in the lamina propria and possibly have a role in allowing T cells access to soluble antigens from the gut lumen (Williams *et al.*, 1992).

Bronchus-associated lymphoid tissue (BALT)

BALT is not thought to be present in humans, and a number of other species (Pabst *et al.*, 1991), and is inconstant in ruminants. Where it is constantly present (rabbits, mice, rats, guinea pigs) it occurs at bronchial and bronchiolar bifurcations. Because of space limitations, the follicles in the lamina propria are often squeezed down through the adjacent cartilaginous rings. Development and amplification appears to be in response to antigenic stimulation, so that fully functional lymphoepithelium appears only after birth (Bienenstock *et al.*, 1982; Bienenstock, 1985).

Conjunctiva-associated lymphoid tissue (CALT)

The existence of CALT has been shown in studies of rabbits, guinea pigs, chickens and turkeys (Axelrod and Chandler, 1979; Chandler and Axelrod, 1980; Franklin and Remus, 1984; Fix and Arp, 1991a and 1991b). In chickens and rabbits the CALT is primarily located in the lower eyelid, and does not develop until several weeks after birth or hatching (Fix and Arp, 1991b). The behaviour of these

epithelial tissues seems to be identical to other MALT, suggesting that they are a functional component of the common mucosal immune system.

MALT and mycobacteria

As early as 1922, Kumagai (1922) realised that mycobacteria, both dead and alive, could be taken up by the follicle-associated epithelial cells of the rabbit intestine in the area of the caecum, without the subsequent development of local lesions. More recently Fujimura (1986) has demonstrated how Bacille Calmette-Guérin (BCG) organisms specifically adhered to rabbit Peyer's patch M-cells within 1 hour of inoculation and were later endocytosed, transported through the cells, and subsequently phagocytosed by macrophages. Similar behaviour has also been observed for the uptake of *M. paratuberculosis* in the intestine of calves (Momotani *et al.*, 1988). Although there is limited evidence for uptake of *M. paratuberculosis* by intestinal epithelial cells in the vicinity of infected M-cells (Garcia Marin *et al.*, 1991), these observations are more likely to have arisen as a consequence of movement of infected macrophages out of Peyer's patches (LeFevre *et al.*, 1978). In humans the most common site of intestinal tuberculosis is in the ileocaecal area, which may be due to a combination of physiological stasis and the availability of a "permissive" epithelium containing a high concentration of Peyer's patches with good exposure of the M cells to the lumen (Bentley and Webster, 1967; Gaffney *et al.*, 1987; Liebler *et al.*, 1995). However, large numbers of mycobacteria are less likely to reach the Peyer's patches than the tonsils, as the environment in the proximal alimentary tract does not favour bacillary survival (Gaudier and Gernez-Rieux, 1962; Talavera *et al.*, 1994).

In man, the tonsil is a frequent site of *M. tuberculosis* infection in people with respiratory involvement, however it is rarely the site of the presenting lesion (Çelik *et al.*, 1995). Calmette (1923), described tuberculous tonsillar lesions in humans as rare, and usually occurring as small, pallid lesions, found at the base of the organ. It has been suggested however that pharyngeal node involvement in humans, may be a consequence of primary tonsillar involvement (Shriner *et al.*, 1992), and Wright (1950) reported that in 50% of cases with pharyngeal node tuberculosis, that the tonsils showed evidence of infection.

In cattle involvement of the oropharyngeal tonsil and Peyer's patch in the acquisition of infection from oral routes of infection was shown clearly by White and Minett (1941) in experiments where contaminated milk was fed to calves. Large doses of bacilli were necessary to establish visible lesions at these sites. In another series of experiments, calves given large oral doses of *M. paratuberculosis* in milk, showed that the oropharyngeal tonsil was the most commonly infected site in the first few days following inoculation (Payne and Rankin, 1961). From the tonsil, the infection spread initially to the medial retropharyngeal lymph node, and thence to other sites. The resultant tonsillar lesions were all microscopic in nature and consisted of small clumps of epithelioid cells.

In another experiment involving feeding *M. bovis* contaminated milk to calves, Edwards (1937) showed that infection established in 23 of 36 calves in the trial, and in all cases the lesions encountered were found in the thoracic cavity only. This provided evidence that infection of the lymphoepithelial tissues of the alimentary tract often occurs without local gross lesion development in the Peyer's patches or the draining lymph nodes, and that haematogenous dissemination to predilection sites occurred commonly. Similar earlier observations had been made in animals by other researchers, in particular, Calmette (1923), and in humans following the Lübeck disaster, in which children were accidentally orally inoculated with virulent *M. tuberculosis* (Anon., 1935). Ulceration of the Peyer's patches apparently occurs only under conditions of extreme bacillary challenge, such as when enormous oral inocula are delivered (Griffith, 1907; White and Minett, 1941), or when large amounts of infected sputum, from pulmonary involvement, is swallowed (Glover, 1941; Bentley and Webster, 1967). In humans, high rates of gastrointestinal tuberculosis are associated with active pulmonary and laryngeal disease. Early or non-progressive GALT lesions in humans have been described as microgranulomas which develop at the periphery of lymphoid follicles (Gaffney *et al.*, 1987). Macroscopic bowel lesions are thought to be induced by large numbers of swallowed bacilli, which are taken up by the lymphoepithelium of the Peyer's patches and mucosal lymphoid aggregates in the large intestine (Carlson and Owen, 1987; Gaffney *et al.*, 1987).

The inoculation of *M. bovis* into the oropharyngeal tonsil of red deer (*Cervus elaphus*) has recently been demonstrated to reproduce clinical disease in these

animals which is indistinguishable from natural cases of the disease, in which the medial retropharyngeal lymph node is most commonly involved site (Mackintosh *et al.*, 1995). In these studies as few as 8 colony forming units (cfu) have been instilled into tonsillar crypts, and produced disease in 50% of the inoculated animals. Infection of the tonsils was associated with spread to, and disease development in the medial retropharyngeal lymph nodes. Gross tuberculous lesions of the tonsils were noted to be uncommon despite the animals being inoculated directly into the crypts (Mackintosh and Griffin, 1994). The medial retropharyngeal node is the commonest site of tuberculous lesions, outside of the thorax, in cattle (Francis, 1958; Crews, 1991; Neill, 1994). This suggests that the acquisition of infection by the tonsil may be one of the more important routes of natural infection in both cattle and deer. Tuberculous lesions of possum oropharyngeal tonsils have also been reported by Cooke *et al.*, (1995) and Jackson, *et al.*, (1995a). The lesions were usually found amongst the lymphoid nodules, but also occasionally extended to involve the lymphoepithelium, where in some instances acid-fast bacilli (AFB) were visible between the epithelial cells.

Racz *et al.* (1978) showed that killed BCG organisms were taken up in BALT of the rabbit, and transported in macrophages to the draining hilar lymph node. The BALT in BCG vaccinated rabbits became more active in antigen transport after the lungs had been challenged with intratracheal BCG (Tenner-Racz *et al.*, 1979). BALT in rats, following intratracheal administration of BCG, showed an increase in the area under the epithelium, a general increase in the number of lymphocytes (without an increase in plasma cells or germinal centres) and more lymphocytes migrating into the epithelium (van der Brugge-Gamelkoom, *et al.*, 1985), thus suggesting that mycobacteria produce an activation of lymphoepithelial tissues, without a marked inflammatory component. *Mycobacterium tuberculosis* has however been shown to produce tubercles in the BALT of guinea pigs following aerosol infection, but only late in the course of disease (Gardner, 1922). It has been proposed that when M-cell possessing BALT is present, this may in fact be the first site that invading bacilli are taken up by the lungs (Bienenstock, *et al.*, 1982).

The instillation of tubercle bacilli onto the eye and conjunctiva has been shown to establish infection in susceptible hosts, with lesions developing in the regional lymph nodes (Calmette, 1923). This suggests that viable airborne mycobacteria

alighting on the surface of the eye are likely to be engulfed by the M-cells of the CALT, and if in sufficient quantity, may subsequently establish infection in the parotid lymph nodes of susceptible species.

As well as being capable of taking up mycobacteria, the lymphoepithelium also seems capable of shedding organisms following a latent period after the initial infection. Ellsworth *et al.* (1980) found that in pigs orally inoculated with *M. avium*, that the organism could be recovered from faeces, swabs of the intestinal mucosa near intact Peyer's patches, and also from the tonsillar surface. The shedding of bacilli was associated with focal non-encapsulated granulomas in the lymphoepithelial tissues, only some of which contained visible AFB. In humans it has also been proposed that the source of AFB in sputa, from radiographically negative cases of pulmonary tuberculosis, is in fact the "endobronchial glands at the minor divisions of the bronchial tree"(=BALT?) (Kent *et al.*, 1967). The discharge of organisms from the surface of MALT is consistent with the observations of Bockman and Stevens (1977, cited by LeFevre *et al.*, 1978) where horseradish peroxidase was shown to undergo bidirectional transport in Peyer's patches.

Of all the mucosal surfaces, only those endowed with a functional lymphoepithelium seem capable of taking up mycobacteria (Figure 2-1). This endocytosis into M-cells occurs rapidly, and serves to present mycobacterial antigen to the underlying lymphoid cells within hours of contact with the bacilli. The mycobacteria in the lymphoepithelial tissues normally do not produce gross lesions, unless the antigenic load is overwhelming, resulting in a breakdown in the epithelium and an outpouring of inflammatory cells to the lumen. The presence of mycobacteria typically stimulates a low grade inflammatory response with the development of small clusters of subepithelial macrophages. Some of these infected macrophages migrate between the M-cells to the lumen of the viscus, and thus provide a route of bacillary excretion. Other infected macrophages will migrate via the lymphatics or bloodstream, thereby acting as "Trojan horses" which disseminate infection to other sites in the body. Free bacilli may be carried in the efferent lymph, or pass through the lymphoepithelium but this is only likely when the mononuclear phagocyte system is overwhelmed by large numbers of bacilli.

Other mucosal surfaces

There has been no demonstrated capacity of the alimentary or respiratory mucosal surfaces to take up mycobacteria in the absence of functional lymphoepithelium. However, large doses of bacilli have been shown to cause ulceration of mucosal surfaces, such as in the nasal cavity or trachea, during experimental infection or in advanced disease (Calmette, 1923; Neill, *et al.*, 1988b).

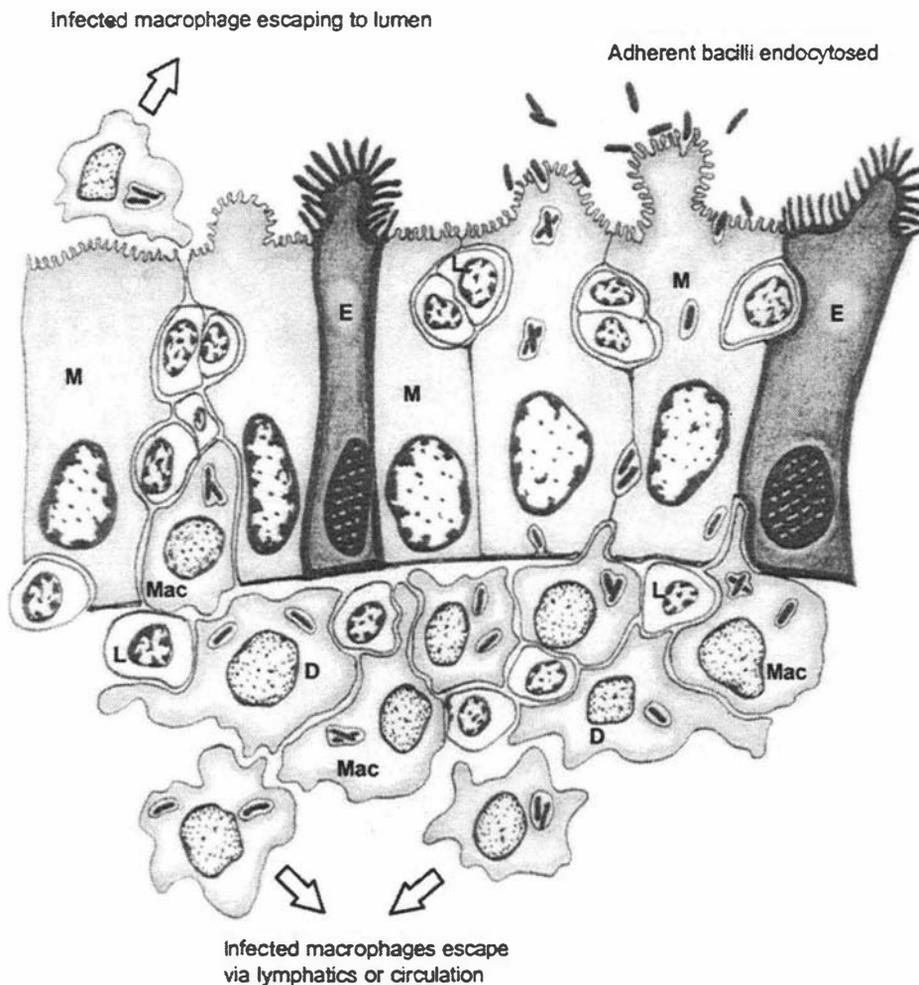


Figure 2-1. Schematic representation of a section of Peyer's patch lymphoepithelium. Mycobacteria free in the intestinal lumen adhere to microfolds and are endocytosed by the M-cells (M), which present bacilli to underlying dendritic cells (D) and macrophages (Mac). These, and accompanying lymphocytes (L), lie in the dome area above the lymphoid follicles. Macrophage traffic may carry the bacilli to other sites in the body, or back through the lymphoepithelium to the mucosal surface. Normal epithelial cells (E) appear to take no part in the processing of particulate antigens

Skin

Experimentally tuberculosis infection can be effected through healthy skin of cattle, guinea pigs and rabbits, by vigorous rubbing, epilation, or after application of bacilli to newly shaven skin (Calmette, 1923). The infection usually leaves no sign on the skin itself, and often produces no disease in the regional lymph node. Disease development at distant sites occurs slowly.

Lungs

The respiratory tract is divided into the upper part containing the nose, pharynx, larynx and the trachea, and the lower portion consisting of the bronchi, bronchioles and lung. Airborne particles may settle on the mucosal surfaces of any part of the respiratory tract, or may, if sufficiently small, penetrate the alveolar spaces. However, particulate antigen, i.e. matter such as tubercle bacilli, can also reach the lung by the bloodstream.

As described earlier, mycobacteria alighting on the nasal mucosa may pass over the nasopharyngeal tonsillar tissues and be engulfed by M-cells. Bacilli which find their way down into the tracheal and bronchial mucosa may also be similarly engulfed by M-cells in animals with functional BALT, or will be almost completely removed from the respiratory tract by muco-ciliary action within 24 hours (Muir, 1972). However, despite these modes of infection being possible, it is generally believed that the principal route of infection for aerosolised bacilli is via entry into the alveolar space, where they are engulfed by pulmonary alveolar macrophages (PAMs) (McMurray, 1994; Dannenberg and Rook, 1994).

Macrophages of the lung originate in the bone marrow, from which they leave as newly formed monocytes in the bloodstream. Differentiation and maturation occur in target organs where they become resident macrophages (histiocytes) with limited ability to replicate in situ (Winkler, 1988). Maturation of monocytes in the lungs produces may produce two cell types, the PAMs and the resident pulmonary intravascular macrophages (PIMs) in some species. Pulmonary alveolar macrophages, which are believed to be the first line of defence against pulmonary mycobacterial invasion are believed to be poor antigen presenters, and more refractory than peripheral blood monocytes to activation (F. Aldwell, pers. comm.). This may partly explain the apparent susceptibility of many species to pulmonary tuberculosis. Alveolar macrophages in new born lambs and pigs initially have

limited phagocytic and bactericidal properties, but these increase with age (Zeidler and Kim, 1985; Weiss *et al.*, 1986). This functional immaturity may explain why the young of some species, such as pigs and deer, have been noted to rapidly develop severe disease following tuberculosis infection. However, it has also been suggested that immature immune systems demonstrate an immunological hyporesponsiveness (Watson and Gill, 1991). In immature ruminants the development of inappropriate immune-tolerance phenomena when exposed to mycobacteria is possibly associated with a high proportion of circulating gamma/delta T lymphocytes (Chiodini and Davis, 1992; Veazey *et al.*, 1994). It is suspected that during the early stage of lung infection mycobacteria are principally resident in PAMs, inside which they replicate, and by mechanisms unknown find their way through the capillary endothelium into the bloodstream and lymphatic vessels where they are probably transported as intracellular parasites within monocytes and macrophages (Dannenberg and Rook, 1994).

Apart from residing in macrophages, mycobacteria may also invade other cell types such as endothelial cells, epithelial cells and fibroblasts (Chan and Kaufmann, 1994). *Mycobacterium tuberculosis* is capable of infecting human type II alveolar pneumocytes, and replicating more freely within these cells than in “professional” phagocytes (Berrnudez and Goodman, 1996; Mehta *et al.*, 1996). This ability of mycobacteria to reside in “non-professional” phagocytes has been hypothesised to allow the bacilli to temporarily evade normal host defenses, as these infected cells may be less capable of antigen presentation, and thus less likely to be initially destroyed by cytotoxic lymphocytes or natural killer cells. However, Chan and Kaufmann (1994) have shown that following a period of infection, that the “non-professional” phagocytes become more susceptible to the cytolytic effects of tumour necrosis factor (TNF). This aspect of bacillary behaviour may thus confer a survival advantage to the organism, as they may be able to replicate freely for a period, before release following TNF- induced cytolysis.

Generation of airborne particles

The ability of livestock or small mammals to generate mycobacteria-infected airborne particles has not been investigated, thus much of the information on respiratory spread amongst animals has been made by inference from human studies, or by aerosol infections of laboratory animals only (Wells and Lurie, 1941;

Lurie *et al.*, 1950; Riley *et al.*, 1959). It is believed that most pulmonary infections arise as a consequence of the inhalation of droplet nuclei. These are formed as evaporation removes water from respiratory droplets, which as a consequence become lighter and smaller than the droplet from which they were derived, and thus can remain suspended in air currents for prolonged periods (Langmuir, 1961).

There have been numerous studies investigating the mechanics of droplet generation and deposition in humans (Duguid, 1946). Duguid (1946) found that most droplet nuclei generated by people through coughing, sneezing and loud talking were in the size range from 0.25 to 42 μm , with most between 1 and 2 μm . However, the few direct observations which have been made on the size of bacteria-laden particles in mouth generated sprays indicate a unimodal size distribution, with the mode in the 10-14 μm range (Williams, 1972). Thus only a small fraction of the aerosolised bacteria derived from human sources are associated with particles having diameters of less than 4 μm , which is approximately the size which is known to penetrate the alveolar spaces in man (Lippmann and Albert, 1969). Sneezing is likely to generate 100 times more droplets than coughing, but is unlikely to result in the expulsion of many infected droplet nuclei of a size which will reach the alveoli, unless enormous numbers of pathogens exist in the saliva at the front of the mouth, where most of the aerosols are formed (Williams, 1972). Theoretically very few people with pulmonary tuberculosis appear capable of generating significant quantities of infected droplet nuclei. To be a significant shedder of viable organisms in droplet nuclei an enormous number of bacilli must be present in the oral cavity and/or upper regions of the respiratory tract. In humans, the density of infectious droplet nuclei in the air is believed to be determined by the frequency of coughing, the density of bacilli in the respiratory secretions and the volume of the air space (Rieder *et al.*, 1989). Although, in referring to humans, Langmuir (1961) states that in practice only a few “tuberculous individuals act as effective disseminators, and these do so probably intermittently and only under certain circumstances”, some individuals have been extremely infectious, especially those with tuberculous laryngitis (O’Grady and Riley, 1963), presumably as a result of aerosol generation from vibrating vocal folds. The infectivity of tuberculous cattle also appears to be intermittent and limited to some individuals (Neill *et al.*, 1989).

It has been argued that some ingested mycobacteria may become aerosolised during the process of mastication or swallowing, these may then be aspirated and establish pulmonary infection (Glover 1941). However, there is no substantial evidence to support such a mode of respiratory infection. Mullenax *et al.* (1964) demonstrated that bacteria from the rumen can penetrate the lungs via the inhalation of eructated gases. Although there were no measurements made on the particle sizes, nor depth of lung penetration in these experiments, this mechanism could potentially provide a route of infection to the lungs following ingestion of *M. bovis*. However, it would seem improbable, that from a small number of bacilli likely to be ingested by a mature ruminant (compared with the enormous numbers of organisms already in the rumen liquor) that any viable tubercle bacilli would find their way into the alveolar spaces.

The quantities of bacteria dispersed from contaminated fur, skin and dust should not be underestimated. People have been shown to liberate 10^6 bacteria-carrying particles whilst undressing and redressing, and many are liberated while exercising naked (Noble and Davies, 1965, cited by Williams, 1972). Dust can also contain viable bacilli for prolonged periods when contaminated by infected sputum (Chaussé, 1913 and 1914, cited by O'Grady and Riley, 1963). Lurie (1944) also believed that dust generated from contaminated, urine soiled rabbit bedding material was the source of airborne bacilli which infected adjacent animals separated by an air space. It is likely that animals in which the disease is characterised by suppurating sinuses, will have extensive skin contamination, and will generate significant quantities of contaminated scurf, hair and dust. Indeed it may be of some significance, that in all experimental infections in possums in which there has been evidence of airborne transmission to uninfected controls, there has been animals present with draining tuberculous sinuses in addition to tuberculous lung lesions (O'Hara *et al.*, 1976; Corner and Presidente, 1981; Isaac *et al.*, 1983; Buddle *et al.*, 1994).

Uptake of airborne particles

Most of the research investigating respiratory infection with airborne particles has been conducted by infecting rabbits and guinea pigs in specially designed chambers (Wells and Lurie, 1940; Lurie *et al.*, 1950; Riley *et al.*, 1959). This work resulted in researchers concluding that small droplet nuclei carrying several bacilli were

necessary to initiate a single primary tubercle in susceptible hosts (Lurie *et al.*, 1950; O'Grady and Riley, 1963; McMurray, 1994).

Studies with radioactive particles in humans (Lippmann and Albert, 1969) have shown that the majority of particles of diameter greater than $4\mu\text{m}$ impact in the nasal or tracheobronchial mucosa and fail to penetrate the alveoli. In humans, few particles greater than $20\mu\text{m}$ in diameter and only about 50% of particles of $5\mu\text{m}$ in diameter are able to penetrate the nasal cavity during breathing at rest, and many particles as small as $1\mu\text{m}$ in diameter are deposited in the nose during normal breathing (Muir, 1972). The predicted optimal particle size for alveolar deposition is in the range of 1.9 to $2.2\mu\text{m}$. Morrow (1980), using a human model of respiratory deposition, predicted that the nasal airways will be the dominant deposition site for virtually all airborne forms of bacteria and fungi. As sputum droplets, fibres and desquamated skin appear to be in the 4 to $10\mu\text{m}$ size range these will also principally impact on the nasal mucosa. The deposition of particles in the nasal cavity of animals may be higher than in humans, due to the relatively larger size of the nasal cavity, differences in structure and the lower likelihood of mouth breathing.

Although it was once thought that deep penetration of bacilli into the alveolar space was necessary for the establishment of tuberculosis from airborne bacilli, this may not be true in all instances. Small aerosolised droplet nuclei $\leq 5\mu\text{m}$ may reach the alveolar space and establish pulmonary infection, whereas larger contaminated particles generated in the mouth or nasal cavity, and those coming from the skin of animals with contaminated body surfaces, will be preferentially trapped in the nasal cavity or large airways where bacilli may be taken up by the nasopharyngeal tonsil, or BALT (if present) or possibly the Peyer's patches after swallowing. Many particles down to $1\mu\text{m}$ in size will also be trapped in the upper airways (Baskerville, 1981).

Although droplet nuclei infection may occur in the lungs of susceptible hosts, macroscopic lesions will not necessarily develop in the pulmonary parenchyma. For example, Riley *et al.* (1959) found with guinea pigs infected by aerosols generated by tuberculous human patients, that despite finding respiratory lymph node involvement in nearly every case, no pulmonary tubercles were found in 20 of 71 tuberculous animals. This suggests that following a low dose alveolar or BALT

infection, that bacilli can be carried rapidly to the regional lymph nodes without producing macroscopic lesions in the lungs. Lesions in the respiratory lymph nodes of cattle and deer are also commonly found in the absence of pulmonary parenchymatous lesions (McIlroy *et al.*, 1986; Hathaway *et al.*, 1994), although detection of small lesions in the lung is notoriously difficult.

Although airborne transmission of tubercle bacilli undoubtedly occurs, the exact process by which any species, other than man, generates infectious aerosols or airborne particles is poorly understood. However, many of the current beliefs on airborne transmission are based solely on extrapolation from human studies of particle generation, and thus should be treated cautiously. Similarly, the fate of inhaled tubercle bacilli, and the establishment of pulmonary lesions, has only been thoroughly studied in laboratory animal models which may have limited applicability to other species which are natural hosts of *M. bovis*, and frequently succumb to pulmonary disease.

Conclusion on routes of infection

The lymphoepithelial tissues are important potential sites for primary infection with tubercle bacilli. Infection at these sites will usually not produce local gross lesions development, but dissemination to other sites is possible, especially if the infecting dose is large, or significant local replication occurs. Although pulmonary alveolar infection from droplet nuclei occurs, the fine detail of this process in domestic stock and most small mammals is poorly understood. Airborne infection need not necessarily establish infection in the alveoli, but may establish via the nasopharyngeal tonsil or BALT. It is thus possible to have pulmonary infection (BALT) without lesion formation in the lung parenchyma itself, and which will allow subsequent lesion development in the respiratory lymph nodes. This may be a possible cause of the enigmatic incomplete primary complexes of the pulmonary tissue in those species with a functional pulmonary lymphoepithelium. Whether or not a particular tissue becomes massively infected with mycobacteria will depend upon many factors, among which are its anatomical location in relation to the point of entry of the organism, the capability of the immunocytes at the primary lesion site, virulence of the organism, and the speed with which the animal can mount a protective immune response.

Haematogenous dissemination of M. bovis within the body

Although it has long been realised that tubercle bacilli disseminate to internal organs via the blood stream, it was only shortly after the turn of the century that, Müller and Ishiwara (1914) cited by Feldman, (1936) isolated tubercle bacilli from the blood of a variety of tuberculous animals. Twelve of 33 (36%) heart blood specimens taken from tuberculous cattle, calves and pigs were found to contain viable bacilli, by means of guinea pig inoculation.

More recently, Barry *et al.* (1993) found evidence for *M. bovis* DNA in the blood of 9/10 reactor cattle subjected to polymerase chain reaction testing (PCR). Most of the samples reacting positively were in the centrifuged cell pellet collected from serum, thus indicating that the DNA was predominantly associated with blood cells or whole bacteria. No blood samples from uninfected animals were examined, so it is possible that the results may have been spurious because of the poor specificity which plagues PCR tests. However, similar findings have been reported in humans. Schluger *et al.* (1994) were able to isolate *M. tuberculosis* DNA from the blood of all their human patients with active pulmonary tuberculosis, but not from healthy controls, including some skin-test positive cases. In another trial, Rolfs *et al.* (1995) also detected mycobacterial DNA in 39 peripheral blood samples from 76 immunocompetent patients with pulmonary tuberculosis, the sensitivity of detection increasing with the severity of the disease process. No mycobacterial DNA was found in 47 control bloods. The conclusion of Rolfs *et al.* (1995) was that “The blood of patients with no trace of extrapulmonary disease or miliary pattern were PCR positive for *M. tuberculosis* supports the hypothesis that *M. tuberculosis* escapes from the alveolar space to the blood circuit more often than previously thought”. Recently PCR has been used to detect *M. paratuberculosis* DNA in the blood of cattle (van der Giessen *et al.*, 1994). Four of five clinical cases were positive, as were another 43 of 72 dairy cattle from an infected herd, in which 21 animals were found to be faecal culture positive. The fact that many of the mycobacteria in the blood are likely to be viable has been shown by Müller and Ishiwara (1914) cited by Feldman (1936), and also more recently by Hanna *et al.* (1995) who found that of 505 bloods from AIDS cases screened for tuberculosis, and which were cultured for mycobacteria, 49 isolates of *M. avium* complex and 3 of *M. tuberculosis* were recovered.

Intracellular pathogens, such as *Salmonella typhimurium*, *Listeria monocytogenes*, *M. bovis* and *M. paratuberculosis* are believed to be transported in the blood within monocytes/macrophages which show “lymphoid homing” mechanisms and other tissue trophic behaviour (Marco *et al.*, 1991; Verjans *et al.*, 1994; Streeter *et al.*, 1995). This carriage of bacilli within monocytes may have been the mechanism which allowed Duchaine (1938) (cited by Francis, 1958) to experimentally demonstrate in rabbits, the accumulation of tubercle bacilli in irritant-induced pleural exudates. Periodic cultures of these exudates (undoubtedly loaded with monocytes), showed that there was a continuous bacillaemia throughout the course of the disease in the subcutaneously inoculated rabbits.

Mycobacterium paratuberculosis infected macrophages have been found to accumulate in the udder of infected cattle prior to calving (Streeter *et al.*, 1995). This accumulation of bacilli in the udder providing further evidence of the carriage of mycobacteria in the bloodstream, even in subclinical cases of disease.

Experimentally, oral administration of BCG produces a transitory bacillaemia for 3 to 5 hours post-inoculation (Calmette, 1933). Payne and Rankin (1961) also showed dissemination of *M. paratuberculosis* to many body sites, including peripheral body nodes, within several days after oral inoculation with 200 mg of bacilli, fed in milk to calves. These studies demonstrate the potential of mycobacteria to disseminate via the bloodstream early in the course of infection. However, in these instances the large number of bacilli administered may have overwhelmed the capacity of macrophages in the lymphoepithelial tissues and draining lymph nodes to contain the bacilli.

It is believed that within a few weeks after primary mycobacterial infection, a few bacilli invariably gain entry to the bloodstream (Rich, 1951; Fok *et al.*, 1976). One common result of this bacillaemia is the development of lesions at predilection sites in other parts of the body (Ho *et al.*, 1978). Fok *et al.*, (1976) showed that after guinea pigs were infected via aerosol, with as few as 3 or 4 organisms, bacillary spread from the primary lung lesions to the draining lymph nodes and spleen was observed as early as 2 weeks following the inoculation. By 3 weeks the other lobes of the lungs were also infected. There is clearly a phase of early haematogenous dissemination from primary lesions following infection with tuberculosis. Pre-existing immunity, induced by vaccination with BCG, reduces the release of bacilli

from primary pulmonary lesions in guinea pigs, and has also been shown to inhibit the replication of bacilli which find their way to other sites, especially other portions of the lungs (Fok *et al.*, 1976).

There is evidence from studies involving injected leucocyte preparations, that white blood cells, including monocytes, are temporarily removed from the circulation by entrapment in the lungs, liver and spleen prior to redistribution (Hay and Cahill, 1982). Experiments in rabbits (Thakur *et al.*, 1977) have also shown that damaged leucocytes are retained in these tissues, and thus the adherence/entrapment of degenerating infected monocytes, could offer one plausible explanation for the high frequency of occurrence of tuberculous lesions in the lung of many species.

Bacilli free in the circulation are likely to be taken up by resident reticulo-endothelial cells of the lung, liver, spleen or bone marrow. The lung of many species, including pigs, cattle, sheep and goats removes more bacteria from the blood than does the liver, spleen, or bone marrow, which have a greater role in dogs, guinea pigs, and rodents (Winkler, 1988). Pulmonary intravascular macrophages are particularly numerous in pigs, and in 30 day old animals PIMs cover 16% of the capillary surface of the lung (Pabst and Binns, 1994). However, rodents have very few PIMs, and following intravenous administration of tubercle bacilli to mice, the ratio of bacilli recovered from the liver, spleen and lung was 500:100:1 (Collins and Montalbino, 1976). This greater role of the PIMs in some species is not entirely dependent upon a higher population of macrophages being present in the lung, compared with Kupfer cells in the liver, but also due to the lung receiving 100% of the cardiac output, compared with 20 to 30% for the liver.

In quoting M'Fadyean, Glover (1941) emphasised the fact that "one ought to avoid the mistake of denying any importance to ingestion, either in man or cattle, or of asserting that tubercle bacilli which enter the body by way of the alimentary canal are never the cause of tuberculosis with lesions apparently primary in the lungs". As discussed earlier, bacilli may enter the body via lymphoepithelial tissues without leaving any macroscopic evidence at the site of entry. Such organisms are likely to disseminate from these primary sites inside macrophages, and may establish lesions in distant locations before the onset of protective immunity prevents further development of gross lesions. The pig serves as a good example of a species in which widespread infection can result from primary alimentary tract uptake of

mycobacteria. Generalised disease, frequently resulting from alimentary lymphoepithelial engulfment of bacilli, may cause prominent lung lesions, especially in younger less resistant animals (Francis, 1958).

As well as local spread via lymphatic vessels, *M. bovis* is capable of widespread dissemination via the circulatory system. This initially occurs early after the primary infection, and continues whilst the disease is active. Lesion development subsequent to this early dissemination will occur in non-immune susceptible hosts, at sites which trap bacilli or take up infected macrophages, and which favour replication of the organism.

Compartmentalisation of the immune response

The differences in the common sites for location of lesions between species i.e. species predilection sites, suggests *inter alia* that the immunocytes of each species may have varying capabilities for controlling the disease process, which is dependent upon the organ compartment concerned. For instance it is well recognised that the predilection sites in rabbits are the lung and kidneys, no matter what the route of infection. When bacilli are injected intravenously into a rabbit, the bulk of the bacilli are taken up by the liver and the spleen, and relatively few by the lung. The liver will subsequently develop small visible lesions, but while lesions of the lungs and kidneys progress, bacillary counts of the liver and spleen fall and lesions of these organs regress and resolve (Soper, 1917; Lurie, 1964). Other examples of lesion site predilection exhibited by mycobacteria have been seen in mice with *M. lepraemurium*, where the organism shows a preference for bone marrow reticulum cells (Brown and Draper, 1976). The kidneys in badgers (*Meles meles*) (Clifton-Hadley *et al.*, 1993), the liver and spleen in guinea pigs (Wilson and Miles, 1964) and the lungs of possums (*Trichosurus vulpecula*) (Jackson *et al.*, 1995a), also appear to be predilection sites in those species.

The differences in organ susceptibility to tubercle bacilli seen after haematogenous dissemination (Rich, 1951) suggests variation in inherent abilities of the organ-specific resident macrophages to destroy the bacilli. However, the progressive increase in bacillary numbers/lesion size during infection indicates that differences arising during the infiltration or maturation of immunocytes may also be involved (Lowrie, 1983). This may be related to inherent, but unknown, characteristics of the compartment or organ involved, which varies the ability of resident immunocytes to

resist the bacilli. For example, Redmond *et al.* (1991) found that the Kupfer cells in the liver of mice were less capable of respiratory burst activity than peritoneal macrophages, a strategy which may prevent unnecessary damage to the adjacent hepatocytes. Activated Kupfer cells were still capable of intracellular parasite control, presumably by alternative mechanisms. Clearly multiple immunomodulatory mechanisms operate simultaneously and independently within specific compartments of the immune system, and these processes must be affected by the physiological system within which they are acting (Lysle and Coussons-Read, 1995). The characteristics of the anatomical environment in which lymphocytes encounter antigen, then lodge after the phase of antigen elimination (or persistence), will influence the phenotype of both the acute phase and memory components of the immune response (Doherty, 1995).

Even within one organ there are apparent differences in the ability of *Mycobacterium* spp. to replicate and produce progressive lesions. Medlar (1940), in an elegant quantitative study of lesion distribution and characteristics in bovine lungs, showed that although all lobes developed primary foci, the most progressive lesions occurred in the dorsal diaphragmatic lobes. Stamp (1948) also found that the most frequent location of tubercles in the lungs of cattle was in the dorsal diaphragmatic lobes, even after allowing for the larger volume of these lobes. Similar observations have been made on tubercles in the lungs of sheep (Davidson *et al.*, 1981). It has also been noted in human pulmonary tuberculosis that cavitory and progressive lesions occur preferentially in the apical lung lobes. Dock (1946) first suggested that this preferential development of lesions in the more elevated lung lobes was due to the higher oxygen tension (pO_2) in these apical lobes, associated with gravitational effects on lung perfusion. These ideas have been strongly supported by results of various experimental studies. Surgical occlusion of a bronchus has been shown to cause almost complete healing of lesions, and creation of left to right shunts in monkeys increases the severity of lesions in arterialised lung segments (Meylan *et al.* 1992). Medlar and Sasano (1935) cited by Meylan *et al.* (1992) showed with rabbits that the normal caudo-dorsal distribution of lung lesions could be shifted to the cranial lobes if the rabbits were restrained head upwards. Sever and Yeomans, (1957) found that housing tuberculous guinea pigs in an atmosphere containing 10% oxygen diminished the extent of tuberculous lesions, whereas enriching the oxygen concentration resulted in increased counts of

mycobacteria in the lung and liver. Rich, (1951) also found that an enriched oxygen supply enhanced bacillary growth in the spleen and liver of laboratory animals. Mycobacteria also survive the longest in peritoneal infections when in proximity to blood vessels (Lack, 1956). The bulk of the evidence in these studies suggests that tissue pO_2 has a significant influence on the progression of tuberculous lesions.

One explanation provided for the occurrence of this affinity of *M. tuberculosis* and *M. bovis* for highly oxygenated tissues, was that mycobacterial growth was enhanced by oxygen availability (Sever and Yeomans, 1957). However, it appears that the growth of *M. tuberculosis* is suboptimal only at pO_2 of well below 40 mm Hg, the venous oxygen pressure that prevails in many tissues (Meylan *et al.*, 1992). The more likely explanation for the observed phenomenon is that the anti-mycobacterial function of immunocytes is compromised in the presence of high pO_2 . Studies have shown that oxygen pressure during culture of macrophages influences their morphological and physiological differentiation (Simon *et al.*, 1973), which may decrease the ability of these phagocytes to mount an effective anti-mycobacterial defence in high pO_2 environments. Significantly reduced growth of mycobacteria has been found in macrophages incubated under low oxygen tension (36 mm Hg), compared with those incubated at 140 mm Hg, similar to the pO_2 found in the apical lung lobes of humans (Meylan *et al.*, 1992).

The exact mechanisms whereby various host tissues demonstrate a strong resistance to the growth of tubercle bacilli is unknown, but apart from the apparent involvement of low pO_2 , pH and iron availability may also play a role (Lowrie, 1983). The lung of many species is a predilection site for the development of tuberculous lesions, not only because it is commonly the site of primary airborne infection, but because local immune responses to mycobacteria, arriving by whatever route, are apparently less effective at controlling infection than elsewhere in the body.

It is conceivable that these tissue preferences of *M. bovis* may extend to include particular lymph nodes within the body, and this variability in secondary lymphoid tissues may depend upon the "background" of cytokine availability or inducibility (Doherty, 1995). Such a phenomenon could potentially provide a plausible explanation for the axillary and inguinal lymph nodes of possums apparently being predilection sites for tuberculous lesions (Jackson *et al.*, 1995a), if a valid

physiological explanation could be found, and why particular lymph nodes of ruminants, such as the caudal cervical, are commonly diseased whereas many others, such as the internal iliac and ischiatic are infrequently involved.

The possibility also exists whereby replication in specific tissue locations will cause preferential selection of particular genotypes of the organism, such that selected strains are better adapted to survival in that particular compartment of the immune system. Collins and Montalbino (1976) have shown that if tubercle bacilli are gathered from the lungs of tuberculous mice, then reinjected intravenously into uninfected mice, they preferentially locate again in the lung of the new host, compared with bacilli harvested from other sites. This hypothesised 'tissue trophism' of selected strains of mycobacteria may explain why there are occasionally clusters of tuberculous livestock with lesions in uncommon sites, and in which there may have been a common source of infection e.g. a run of parotid lesions in a group of reactor cattle, or a group of deer with joint infections (Hutton, 1979).

These various observations above suggest a dynamic balance between the host's capacity to eliminate the bacilli, and the ability of the bacteria to interact with the host's response in a way which facilitates establishment in the tissues. However, cellular events are also dependent upon the site of localisation, which will further determine the course of lesion development.

Lesion resolution and bacillary dormancy

Part of the historical mystique which has developed around tuberculosis has involved the hypothesis of a specialised "tuberculous granulation tissue" which behaves differently to similar reactions caused by other organisms. The existence of such specialised tissue in humans was dismissed by Rich and McCordock (1929) who state that "repair in tuberculosis is no different to repair elsewhere, except in so far as it is interfered with by the infection". These authors believed that tubercles can resolve completely, especially if not complicated by the appearance of connective tissue in the lesions. Following experience gained through human radiography, Rich (1951) found that not only may small epithelioid tubercles resolve, but that large tubercles with central caseation and encapsulating fibrous tissue and mineralisation, can also disappear leaving only microscopically visible traces of their existence. Both Stamp (1944) and Smith and Jones (1961), when

referring to tuberculosis in animals, also believed that if the bacteria in lesions were eventually overcome, that the tubercles would be reduced to a small mass of fibrous or hyaline scar tissue, similar to that which has been observed in human lungs. The resolution of small pulmonary tubercles in guinea pigs is also described by Gardner (1922), who found that even in tubercles with fibrous encapsulation that “complete restitution” to normal lung tissue was possible.

Rhodococcus equi infection in foals causes a disease very similar to pulmonary tuberculosis in other species, the lesions characteristically being large pyogranulomatous abscesses of the parenchyma and respiratory lymph nodes. Despite radiographic evidence of the lesions being large, multiple and severe, foals are well known to recover completely, such that no gross or histological evidence of disease remains at necropsy, some months following treatment (Hillidge and Zertuche, 1987). These observations on a disease caused by an organism closely related to mycobacteria raises the possibility that resolution of some tuberculous lesions may occur in animals, an aspect of the pathogenesis of disease which has received little research attention over many years of veterinary investigation.

Prior to the treatment of human tuberculosis with drugs, up to one third of all cases became bacteriologically (sputum) negative, and had apparently recovered from the disease when followed up 1.5 to 5 years after initial diagnosis (Grzybowski and Enarson, 1978). It is well recognised in humans that tuberculosis, after initial infection and successful treatment, will remain quiescent, sometimes for many years, before causing a relapse to clinical disease in a proportion of cases. Calmette (1923) citing Loomis (1890) and Pizzini (1892) described prevalences of 26 and 42% respectively, of tuberculosis infection in non-lesioned human cadavers, a finding which also supports the notion of complete resolution in at least some human cases. Reactivation in people may be induced by a decrease in cell-mediated immunity, such as that produced by Acquired Immunodeficiency Syndrome (AIDS) (Wiegshauss *et al.*, 1989; Selwyn *et al.*, 1989). Restriction fragment length polymorphism studies have shown that the relapses in treated patients have been caused by reactivation of the same bacteria which caused the initial clinical disease (Das *et al.*, 1992), thus suggesting that the bacilli have lain dormant or inactive for a lengthy period, and that the disease has not been caused by superinfection with another organism.

Early this century Calmette (1923) eloquently described the resolution of small caseous tubercles and the fate of intra-lesional bacilli. In his words “the cells surrounding the small mass become organised into fibrous tissue, forming a dense wall which thickens gradually up to the centre, where ultimately only leucocytic debris is to be found in the sclerotic framework. A few granular malformed bacilli persist there for years in a dormant state capable of being revived by experimental inoculation after the enveloping substance has been crushed, but ordinarily incapable of multiplying in situ in the lesion itself. In this manner the process of spontaneous healing of the tubercles is most often accomplished, - an apparent healing, rarely complete, since it is only exceptionally that some vestiges of caseous matter and a few bacilli do not persist indefinitely at the centre”. In species where extensive fibrosis, and calcification are not hallmarks of the disease process (e.g. deer and possums) resolution of lesions following the induction of protective immunity, could potentially be an uncomplicated and rapid process, whereby lesion regression/resolution, as observed in some *Rhodococcus equi* infected foals, may be possible.

The malformed dormant bacilli noted by Calmette may be similar to the sterile acid-fast forms (similar to bacilli) which were seen by McCune *et al.*, (1966a) in infected mice treated with antimicrobials, or to the gram-positive, non-acid fast forms reported in sheep lesions by Cordes *et al.*, (1981), and the pleomorphic non-acid fast forms (Much granules) reviewed by Stanford (1987). Variability in the morphology and staining of mycobacteria in tissues and culture media is a well recognised phenomenon (Barksdale and Kim, 1977). The classical tubercle bacillus is thus only one of the many forms of this very plastic microorganism which can readily adapt to unfavourable conditions (Schmelev, 1970).

Recently it has been shown that mice inoculated with *M. tuberculosis*, and subsequently treated by chemotherapy to produce animals from which the organism cannot be cultured, nor made visible by acid-fast stains, still contain substantial amounts of mycobacterial DNA in lung and splenic tissues (de Wit *et al.*, 1995). The presence of non acid-fast *M. paratuberculosis* antigen has been identified histochemically, in recently acquired Peyer’s patch infections in sheep (Garcia Marin *et al.*, 1991), and Condrón *et al.* (1994) have demonstrated the presence of cell wall deficient, non acid-fast *M. paratuberculosis* in macrophages and giant cells

of cattle with paucibacillary Johne's disease. Thus the mycobacterial DNA found in mice by de Wit *et al.* (1995) is possibly due to the presence of dormant/cell wall deficient bacilli with limited pathogenicity, but which may later cause endogenous reactivation of disease. In mouse models the recovery of virulence was found to be delayed by immunity, and it has also been found that administration of high doses of glucocorticoids increased the number of positive cultures from mice in the period post-chemotherapy (McCune *et al.*, 1966a). The factors initiating and sustaining the state of dormancy are unknown, but it has been hypothesised that starvation of nutrients may be a key factor. Tubercle bacilli deprived of nutrients *in vitro* have survived as non-stainable elements for at least 2 years (Nyka, 1974). It is also believed that the loss of acid-fastness is due to the loss of cell walls (Barksdale and Kim, 1977), or the development of microspores, and that these bacilli, known as L-forms or spheroplasts (Fedoseev *et al.*, 1985; Grange, 1996), may be more capable of resisting attack by the host defences, and surviving in protected environments, than normal bacilli (Lowrie, 1983).

Stress and tuberculosis

Stress and immunosuppression

There is general agreement that there is a complex mechanism of interaction between the central nervous system, the immune system and the endocrine system, which when disturbed by stressful stimuli orchestrates a response designed to maintain the homeostatic mechanisms of the individual (Bonneau *et al.*, 1990). Experimental and clinical studies demonstrate that both laboratory and natural stressors alter the activities of lymphocytes and macrophages in a complex way that depends upon the type of immune response, characteristics of the stressor, compartment of the immune system studied, and the timing of the stress relative to the immune response (Dantzer and Kelley, 1989; Lysle and Coussons-Read, 1995). The influence of stress on immunity is mediated not only by glucocorticoids (GC), but also by catecholamines, endogenous opioids and pituitary hormones, such as adrenocorticotrophin (ACTH), arginine vasopressin (AVP = antidiuretic hormone), prolactin and growth hormone (Griffin, 1989; Lysle and Coussons-Read, 1995), so that in any one compartment of the immune system multiple immunomodulatory mechanisms will be operating simultaneously. The connections and feedback loops between the immune system, the brain and nervous system, and the endocrine system is also part of a complex mechanism co-ordinating behavioural and

physiological responses to infection and inflammation. The enormous complexity of the observed physiological effects of stress does not facilitate a functional interpretation of the results on the immune system. This issue requires more focused studies using a single animal species, one particular compartment of the immune system under study e.g. lung or blood, and one pathogen (if any are involved) (Dantzer and Kelley, 1989; Rabin *et al.*, 1989).

Control of glucocorticoid release

One of the most commonly studied changes in the homeostasis of normal body function following a stressor, is those associated with the behaviour of the hypothalamic-pituitary-adrenocortical (HPA) axis. The hypothalamus is considered the efferent arm of the visceral brain. It controls the secretion of various peptide hormones including, corticotrophin-releasing hormone (CRH), AVP and oxytocin, each of which is independently capable of increasing the release of ACTH from the pituitary gland, but which in concert act synergistically (Baxter and Tyrrell, 1986; Khansari *et al.*, 1990; Harbuz and Lightman, 1992). It is as a response to circulating ACTH, that GC are released from the adrenal cortex (Breazile, 1988).

CRH is the most potent stimulator of ACTH and β -endorphin secretion from the pituitary gland. However secretion is also partially controlled by other peptides, including: AVP; oxytocin; angiotensin II; vasoactive intestinal peptide; and serotonin; and in the rat adrenaline and noradrenaline (Johnson *et al.*, 1992). In addition to hypothalamic control of ACTH release in response to stressful stimuli, certain lymphoid organs also have regulatory control over this hormone. Thymic peptides and interleukin-1 (IL-1) have also been reported to regulate ACTH release (Khansari, 1990). Release of GC results in negative feedback, such that elevated GC are inhibitory to the further release of ACTH. This mechanism acts through at least two sites i.e. directly on the pituitary and also on the release of CRH from the hypothalamus.

A high proportion of circulating GC is in bound form, attached to the alpha globulin called "transcortin" or "corticosteroid binding globulin" (CBG). The unbound fraction is considered physiologically active and is believed to exert negative feedback effects on ACTH and CRH release (Johnson *et al.*, 1992).

The response of the pituitary to a variety of stressors is stimulus-specific and dependent upon which ACTH-releasing factors are secreted from the hypothalamus (Harbuz and Lightman, 1992). Stress has been shown to increase the production of certain neuropeptides, among which are ACTH, β -endorphin and N-proopiomelanocortin (N-POMC), all derivatives of proopiomelanocortin (POMC), and all co-released from the pituitary gland. Both ACTH and N-POMC have actions on the adrenal gland. ACTH stimulates steroidogenesis and production of glucocorticoids (GC), whereas N-POMC promotes the synthesis of ribonucleic acid (RNA) by the adrenal cortical cells and is apparently necessary to prevent atrophy of the adrenal cortex (Breazile, 1988).

CRH release is inhibited by ACTH, GC and endogenous opioids, whereas IL-1, IL-2, IL-6, platelet activating factor and TNF, all produced by immunocytes, appear to stimulate the secretion of CRH (Brown *et al.*, 1993). Apart from a cascade of hormonal effects resulting from the release of CRH, this peptide is also thought to be directly responsible for the characteristic behavioural arousal and appetite depression associated with stress. Stressors also promote the hypothalamic release of AVP, oxytocin, growth hormone and prolactin. CRH stimulates release of β -endorphin and dynorphin may mediate the effects of increased CRH release on gonadotrophin secretion, which results in a lowered levels of luteinising hormone (LH) (Williams *et al.*, 1990; Norman, 1993).

Lymphocytes and macrophages are also capable of producing and releasing ACTH and are subject to stimulation by CRH and AVP, and to negative feedback by GC (Blalock, 1989).

Chronic stress

Chronic stress induces a state of lowered responsiveness to physiologic increases in plasma GC, and increased secretion of CRH and AVP (Johnson *et al.*, 1992). The adrenal cortex hypertrophies rapidly in response to chronic stress, but this is not thought to be a direct response to elevated levels of ACTH (Vernikos *et al.*, 1982). It may be a response to elevated levels of N-POMC (Breazile, 1988). With repeated stress there is habituation or adaptation of the HPA axis, resulting in attenuated responses to that stressor. Glucocorticoid and ACTH levels initially rise after the application of the stressor, but fall back to normal or near normal levels as the stress continues, but the normal diurnal rhythmicity in GC release is reduced (Harbuz and

Lightman, 1992). The return to normal circulating ACTH and GC levels, despite the continued presence of the chronic stressor, is thought to be mediated through a decrease in CRH receptors in the pituitary gland (Breazile, 1988). However, the habituation is stressor specific, such that the application of a superimposed novel noxious stimulus will normally elicit an enhanced acute stress response, with higher and more persistent circulating GC levels than usual (Sakellaris and Venikos-Danellis, 1975; Vernikos *et al.*, 1982; Jensen *et al.*, 1995; Hanlon *et al.*, 1995). This enhanced response is believed to be mediated by the release of AVP, rather than CRH, and is possibly associated with a decrease in GC receptors in the hypothalamus, thereby reducing the negative feedback on ACTH release (Sapolsky and McEwen, 1985). In chronic stress there may also be persistently elevated levels of CRH, oxytocin and AVP in the hypothalamus, and increased production of catecholamines and enkephalin by the adrenal medulla.

Actions of glucocorticoids

The physiological actions of GC which are involved in the “conservation-withdrawal” reaction to stress include: 1) anti-inflammatory properties; 2) immunosuppression; 3) regulation of carbohydrate metabolism- gluconeogenesis; 4) enhanced excretion of water load; 5) induction of various enzymes; 6) inhibition of other hormones, some neuropeptides and lymphokines (Kreeger, 1988). Although these actions may have beneficial effects over the short term, there is general agreement that chronic stress is detrimental to the well being of an individual.

Although not mediated by elevated GC levels alone, there are three major pathological consequences of chronic stress on an animal. The first of these is weight loss or reduced growth rate induced by catabolism of body tissues and loss of appetite, water loss, decreased fat deposition and alterations to protein metabolism. Chronic stress also inhibits gonadotrophin release, thus decreasing gonadal function, causing delayed puberty, lack of behavioural receptivity or libido, failure of ovulation and implantation or spermatogenesis and spontaneous abortion. Low birth weights, and decreased offspring survival have also been observed in stressed females (Kreeger, 1988; Johnson *et al.*, 1992).

The other major effect is one of immunosuppression. That this effect of stress is mediated by GC is widely held. However, the finding that in response to stress that adrenalectomised rats show immunosuppression, strongly suggests that other factors

are also involved (Keller *et al.*, 1983). Many other mediators of immunosuppression have been suggested, but evidence for their effects is not strong. Current data suggests that IL-1 acts on the brain to suppress the immune response by activating both the HPA axis and the sympathetic nervous system via hypothalamic CRH (Harbuz and Lightman, 1992). CRH itself may have local actions at the site of inflammatory responses, and may directly alter the production of immunoregulatory cytokines. There is also some evidence to suggest that IL-2, IL-4 and IL-6 may also have immunoregulatory roles involving the HPA axis (Harbuz and Lightman, 1992). Catecholamines are also capable of inducing ACTH release by direct action on the hypothalamus. ACTH is in its own right suppressive of immunoglobulin production and interferon gamma (IFN- γ) synthesis, and also reduces macrophage activation by IFN- γ (Johnson *et al.*, 1984; Blalock, 1989). IFN- γ can also stimulate GC secretion in infected animals in the absence of ACTH, and thus amplify the normal stress response to infectious agents (Breazile, 1988).

The elevation of circulating glucocorticoids has a potent effect on the behaviour of the immune system. They depress the activity of T-lymphocytes substantially, more so than B-cells (Stevenson *et al.*, 1989). Lymphocyte traffic is diminished and T-suppressor cell function is enhanced. The thymus involutes and there is loss of splenic and lymph node mass. Monocyte and macrophage activity, and cytokine production is suppressed by GC, and also results in decreased MHC Class II molecule expression on the surface of these antigen-presenting cells (Snyder and Unanue, 1982; Brown *et al.*, 1993; Hill *et al.*, 1995). Thus some abnormalities observed in T cells after elevation of GC levels can be directly attributed to alterations in macrophage function.

High levels of GC also produce lymphopaenia, eosinopaenia and neutrophilia (stress leucogram). The neutrophilia is induced by the input of mature neutrophils from the bone marrow storage pool, decreased egress of neutrophils into the tissues, and reduced margination. Eosinophils migrate to lymphoid tissues and lymphocytes are redistributed to other compartments of the immune system and immature or selected T cell subsets are lysed (Cupps and Fauci, 1982; Roth *et al.*, 1982; Doherty *et al.*, 1995).

The production of the lymphokines, IL-1, IL-2, tumour necrosis factor, and IFN- γ , all important to cell mediated immune responses, is also depressed by GC (Cupps and Fauci, 1982; Roth *et al.*, 1982; Blecha and Baker, 1986; Johnson *et al.*, 1992; Wallgren *et al.*, 1994).

Effects of selected stressors

Experimentally there have been many types of stressors used to study responses in a variety of animal systems. These have varied from noxious treatments such as foot-shocking of rodents, through to the more physiologic interventions such as social stressors involving the mixing of livestock. A small selection of environmental stressors and their physiological effects are presented below.

Acute food deprivation is known to produce significant elevations of circulating GC in humans and horses (Messer *et al.*, 1995). Chronic protein-energy malnutrition also elevates circulating GC in mice (Hill *et al.*, 1995). These elevated levels impair macrophage function, and are thought to be a significant cause of the immunosuppression induced by malnutrition. In malnourished children and swine free cortisol levels are also high, with the increase in GC exacerbated by decreased levels of circulating CBG (Samuel *et al.*, 1976). Protein malnutrition in rabbits has been shown to cause a decrease in lymphocyte mitogenic responses which was thought to be due to decreased cytokine activity (Bell *et al.*, 1986).

Crowding stress in mice has been shown to principally affect the T-helper cells, in which mitogenic responses were depressed, and the quantity of IL-2 produced was decreased (Rabin *et al.*, 1989). However, these changes were independent of elevated serum GC levels, a finding also reported by Stein *et al.* (1985). In red deer under social stress, circulating GC levels and lymphocyte mitogenic responses were similarly found to be depressed, but antibody production remained normal, and plasma GC response to ACTH was enhanced (Hanlon *et al.*, 1995).

Both heat (35°C) and cold (4°C) stress in mice has been shown to reduce the number and activity of natural killer (NK) cells, and has been associated with increased levels of adrenal cortisol (Won and Lin, 1995). Plasma GC levels in rats and cattle rise rapidly following exposure to high temperatures and thereafter subside over a

number of days (Maickel *et al.*, 1967; Elvinger *et al.*, 1992), thus suggesting that exposure to heat stress produces responses typical of other chronic stressful stimuli.

Sheep suffering from acute heat stress (Minton and Blecha, 1990) show transient increases in cortisol concentration, but these were apparently insufficient to reduce LTA responses. However, chronically heat-stressed sheep and pigs have been shown to have reduced lymphocyte mitogenic responses (Niwano *et al.*, 1990; Becker and Misfeldt, 1995), whereas heat stressed calves showed no evidence of effect on LTA responses. These experiments showed that the response to elevated temperatures varies between species, and furthermore that serum from heat stress-responsive animals induced a suppression of lymphocyte mitogenesis, which was independent of circulating GC and IL-2 concentrations. Heat stress has recently been shown to decrease the numbers of circulating $\gamma\delta$ T cells, which are believed to be important in establishing primary immune responses to pathogens, including mycobacteria (Ferrick *et al.*, 1995; Pollock *et al.*, 1996; Morrow-Tesch *et al.*, 1996).

Glucocorticoids have been shown to reduce T-cell mitogenesis induced by Concanavalin A (Con A) and phytohaemagglutinin (PHA), in both pigs and cattle, the mechanism proposed being through a reduction in IL-2 synthesis (Blecha and Baker, 1986; Wallgren *et al.*, 1994). Although foot-shocking has been shown to increase circulating GC levels in rats, animals which had been previously adrenalectomised still showed significant reductions in lymphocyte mitogenic responses suggesting that mechanisms other than elevated GC were responsible for the effects on lymphocytes (Keller *et al.*, 1983; Kusnecov *et al.*, 1995). Increased Con A responses have been seen in rat lymphocytes exposed to elevated GC levels following emotional stress, thus adding support to the hypothesis that GC do not necessarily cause a reduction in lymphocyte mitogenesis (Croiset *et al.*, 1987). An alternative mechanism whereby lymphocyte mitogenesis may be influenced, is through the endorphins, which have been reported to decrease the mitogenic response of human lymphocytes (Rabin *et al.*, 1989). However, this may not be the same in all species, nor for each of the endorphins or compartments of the immune system (Griffin, 1989).

The results of these experiments suggest that there are significant immunomodulatory effects of stress which do not act through the release of GC. Monitoring peripheral lymphocyte mitogenic responses, or GC levels after a

standardised stressor or ACTH test are clearly satisfactory methods of assessing the immunosuppressive effect of a chronic stressor.

Glucocorticoids and inflammatory responses

Infection with foreign agents or with non-replicating antigens has long been known to induce a classical stress response involving elevations of GC. It is believed that this response serves to reduce the damaging effects of inflammation, which if unchecked, may cause more harm than the antigen itself. Glucocorticoid release may be mediated directly by the release of ACTH from immunocytes, and indirectly by the action of cytokines on the CNS. IL-1 which is released during inflammatory responses has been shown to both increase both plasma ACTH and GC when injected systemically (Dantzer and Kelley, 1989).

There is evidence that the magnitude of an immune response may be limited by the GC that are released as a consequence of the immune response itself, and that these will be governed to some extent by the genotype of the animal (Mason, 1991). Studies of animals which show higher innate stress responses, or which are pre-stressed prior to an inflammatory response have shown that they are better able to curtail the detrimental effects of their own bodies inflammatory processes and will thus be less susceptible to autoimmune diseases (Mason, 1991).

In vivo delayed-type hypersensitivity (DTH) responses, an expression of CMI capability, has in several species been shown to be reduced in response to both heat, cold, and restraint stress. The impact of heat stress on mice (Pitkin, 1965), was first shown to cause a reduction in the DTH response. However, later reports found more complex effects of temperature stress. Blecha *et al.* (1982) found that the induction of the DTH to sheep red blood cells in mice was enhanced by heat stress and either enhanced or suppressed by cold stress, depending upon the timing of the stress relative to the induction or expression of the CMI response. Restraint stress applied before or after induction of DTH has also been shown to reduce the severity of the reaction (Blecha *et al.*, 1982; Okimura *et al.*, 1986).

Administration of systemic dexamethasone to tuberculin-sensitized cattle led to a decreased dermal DTH reaction (Doherty *et al.*, 1995). This reduction was associated with less of certain T cells clones at the reaction site and in the circulation. Fewer cells expressing IL-2 receptors were present at the reaction site

in treated animals, and *in vitro* production of IFN- γ was reduced in peripheral lymphocytes incubated with bovine PPD. There was also evidence that dexamethasone altered the immunological equilibrium in favour T - suppressor cells. In another trial, calves which were administered dexamethasone, or transported long distances by truck showed decreased IL-2 receptor expression of peripheral blood mononuclear cells, which correlated with decreased expression of lymphocyte mitogenic responses to Con A and PHA (Lan *et al.*, 1995). There was also a suggestion from this study that decreased production of IL-12, which is required for clonal expansion of T lymphocytes and NK cells, may also have been involved in reducing the mitogenic responses.

Some stressors are capable of reducing lymphocyte mitogenic responses through interference with production of lymphokines necessary for normal mitogenic responses, and by reducing the ability of the mononuclear cells to bind cytokines. Glucocorticoids clearly have some effect, but the reduction in mitogenic responses seems to be largely independent of circulating GC levels, but may be sensitive to sympathetic nervous stimulation (Bourne *et al.*, 1974; Mormede, *et al.*, 1988; Zakowski, 1995).

Stress and infectious disease

It has been hypothesised that the immune system may serve the role of a non-cognitive sensory system which sends messages to the neural system by cytokine release. If the set of cytokines which are released is stimulus-sensitive, then the pathophysiology that is associated with a particular infectious agent could be related to the set of cytokines released by immunocytes (Blalock, 1989). The cytokine IL-4 and IL-10 are known to be produced by T-helper 2 clones which are functionally specialised and promote B cell activity, whereas IL-2 and IFN- γ are produced by T-helper 1 clones which are known to promote T cell activity (Mason, 1991). These two clones are thought to be mutually antagonistic, each impeding the development of the other (Mosmann and Coffman, 1987). Such reciprocal actions may explain why the magnitude of cell-mediated and humoral responses to an antigen sometimes show a converse relationship (Parish and Liew, 1972). GC have been shown *in vitro* and *in vivo* to promote the production of IL-4 and suppress the production of IL-2 (Daynes and Araneo, 1989), which suggests that GC may promote humoral immunity and antibody synthesis, at the expense of an adequate cell mediated

response. GC may, by their action on $\gamma\delta$ T cells, in fact be able to tip the balance between cell-mediated and humoral immunity in favour of the humoral responses (Mason, 1991; Ferrick *et al.*, 1995; Pollock *et al.*, 1996).

As outlined in the earlier sections GC tend to exert immunosuppressive effects, especially on the cell-mediated immune system. The administration of GC to animals of many different species has been shown to markedly increase their susceptibility to a variety of infectious diseases and cause activation of latent infection. In cattle exogenous GC have been shown to decrease resistance to infectious bovine rhinotracheitis virus, coccidiosis, herpesvirus, bovine viral diarrhoea and parasites (Griffin *et al.*, 1988b).

However stress responses may be immunopotentiating, and increase disease resistance in some circumstances, but this is pathogen dependent. In fowls, for instance, it has been shown that elevated GC levels induced by social stress, increased resistance to *E. coli* and *Staphylococcus aureus* infection, but reduced the resistance to *Mycoplasma gallisepticum*, Marek's disease and Newcastle disease virus (Gross and Colmano, 1967; Gross and Colmano, 1969; Gross, 1972).

In other diseases, such as shipping fever in cattle, salmonellosis in horses and mice, and early lactation infections in dairy cattle, yersiniosis in deer and goats, streptococcosis in opossums (*Didelphis virginiana*), stress has been suggested to have a causal role (Sherwood *et al.*, 1968; Edwards and Dean, 1977; Buddle *et al.*, 1988; Griffin, 1989; Griffin *et al.*, 1990).

The virulence of the organism has been shown to alter the outcome following infection in stressed animals. Previte and Berry (1962) found that in mice, prior exposure to cold temperature stress, before inoculation with low virulence strains of *S. aureus* and *S. typhimurium*, increased the mortality significantly, compared with mice housed under warm conditions. However, cold stress did not affect the mortality rate in mice inoculated with virulent strains of the bacteria. This suggested that the effect of stress could be more critical to the outcome of infections with opportunistic or insidious pathogens.

Where haematological changes in young deer following weaning, cold stressful weather and feed changes were monitored over a 2 month period (Griffin *et al.*, 1988b), the findings suggested that these stressful events were immunosuppressive.

Changes included reduced lymphocyte mitogenic activity, and decreased lymphocyte responses to bovine PPD following vaccination with BCG. Dexamethasone administered to deer has also been shown to induce a state of immunosuppression. Monitoring showed typical changes in haematological profiles, depressed lymphocyte responses to Con A, reduced responses to vaccination with *M. paratuberculosis*, and greater severity of tuberculosis induced by inoculation with *M. bovis* (Thomson *et al.*, 1994). Thus in deer, GC appear to be important in the aetiology of stress responses.

Pigs have also have disease susceptibility increased by temperature stress. Shimizu *et al.* (1978) have shown a direct link between temperature fluctuations and the susceptibility of pigs to transmissible gastroenteritis. In pigs exposed to changes in temperature around the time of infection, and in those exposed to constant low temperatures, there was a greater likelihood of developing clinical disease.

Kelley *et al.* (1982) exposed 3 week-old calves to 2 week periods of both heat and cold stress. Heat exposure significantly reduced the DTH reaction to PPD by 42%, independent of the length of exposure, whereas the DTH reaction to cold (5°C) was increased by 42% above control levels after 1 week, but was reduced by 14% after 2 weeks. The intradermal tuberculin skin test sensitivity is apparently lower in deer recently subjected to stress of captivity, than in deer adapted to farming (Griffin, 1989).

In some small dasyurid marsupials, such as *Antechinus spp.* and *Phascogale calura*, there is an acute stress response in the males, coinciding with the breeding season and increased levels of testosterone (Lee *et al.*, 1977; McDonald *et al.*, 1986; Bradley, 1990). This stress response is associated with increased aggression, general activity and sexual encounters. The outcome is invariably the mass death of males, and has been associated with recrudescence of latent *Babesia spp.* or *Listeria monocytogenes* infections, and gastro-duodenal ulceration. Immunosuppression and other stress effects are associated with elevated free GC levels, decreases in CBG, and a failure of normal GC feedback mechanisms (Bradley, 1990). Stress of captivity has also been implicated in the cause of acute salmonellosis, nematode problems, gastric ulceration and starvation in possums (*Trichosurus vulpecula*) (Keber, 1979; Presidente, 1982).

Genetic resistance mechanisms have been shown to play a role in the susceptibility to intracellular parasites. There is compelling evidence in mouse models that resistance to *Salmonella typhimurium*, *Leishmania donovani*, and BCG is under monogenic control (Skamene, 1985). This gene has been designated as the Bcg gene or Nramp (natural resistance-associated macrophage protein). Experimental evidence suggests that the Nramp phenotype is mediated by macrophages, and provides innate resistance to initial infection (Chan and Kaufmann, 1994). Equivalent findings, suggesting the existence of Nramp genes, have been identified recently in cattle and deer (Frelief *et al.*, 1990; Qureshi *et al.*, 1996; Mackintosh *et al.*, 1995).

Resistant mice, designated Bcg^r, whose macrophages demonstrate enhanced ability to kill organisms in phagolysosomes, and are also able to persistently present MHC class II glycoproteins to T cells, have been shown to be resistant to the effects of GC elevations (Zwilling *et al.*, 1990). However, Bcg^s (susceptible) mice suffered a reduction in MHC class II expression as a result of the applied stress. This suggests that animals with a genotype conferring resistance to intracellular parasites such as mycobacteria, brucellae, salmonellae, and leishmania, will be less susceptible to the effects of elevated levels of circulating GC.

Collectively, these data suggest that the immune system is subject to regulation by environmental factors, such as social and temperature stresses, and poor nutrition, and that the direction and magnitude of the changes may be dependent upon the duration and intensity of the exposure. Furthermore there will be a substantial genetic component which ensures that not only species, but individuals within species, have varied abilities to withstand the adverse effects of stress on the immune system.

Glucocorticoids and the pathogenesis of tuberculosis

Glucocorticoids and tuberculosis

In the early days of GC treatment of tuberculosis in humans, prior to the use of effective chemotherapy, their use was often accompanied by the development of florid tuberculosis (Horne, 1966). This prompted the American Trudeau Society (1952) to state that “even in the absence of known tuberculous infection, roentgenographic examination of the chest before, during and after hormone (GC) therapy is wise since there may be exacerbation of unsuspected tuberculous

lesions". There is convincing evidence that GC, and to a lesser extent ACTH, adversely affect tuberculosis in experimental animals (Le Maistre *et al.*, 1953; Home, 1966) and even animals which normally show extreme resistance to the disease, such as rats and mice, may succumb to disseminated disease when treated with GC (Michael *et al.*, 1950; McCune *et al.*, 1966). Thomson *et al.* (1994) have also demonstrated increased susceptibility to bovine tuberculosis in deer treated with dexamethasone.

Action on macrophages

Early animal research into the effect of GC on the response to infection with tubercle bacilli in rabbits showed that administration decreased the inflammatory response, such that typical tuberculous granulation with well developed caseation was absent in treated animals. Under the influence of large doses of GC, macrophages were shown to lose their ability to destroy engulfed bacilli (Lurie and Zappasodi, 1955), and that the maturation of epithelioid cells (activated macrophages) is retarded (Lurie, 1955).

Several more recent reports have shown that exogenous GC suppress the antimicrobial activity of macrophages and exacerbates the growth of mycobacteria (Schaffner, 1985; Cox *et al.*, 1989). This in part may be due to a reduced production of IFN- γ , the production of which in tuberculous cattle is reduced after dexamethasone administration (Lawler Goff, 1996). Rook *et al.* (1987) have also found that the ability of dexamethasone to suppress antimicrobial activity of macrophages varied between individuals, which suggested a possible genetic difference in the effect of GC on macrophages.

Stressors and tuberculosis

As early as 1919, Ishigami (1919) (cited by Khansari *et al.*, 1990) found that phagocytic activity of macrophages towards tubercle bacilli decreased during episodes of emotional stress, which led him to postulate that a stressful life led to immunodepression and increased susceptibility to tuberculosis.

In the early 1900s, South African natives who were suffering physical and emotional stress after commencing work in the Rand mines, were believed to have a high incidence of tuberculosis due to the reactivation of latent tuberculosis (Francis, 1958). Seventy two percent of the workers were tuberculin skin test positive when

starting work at the mines, and of those who progressed to clinical tuberculosis status, 70% died, a higher proportion than in similarly affected Europeans. Upon return to the Kraals, many natives without severe disease apparently effected clinical recoveries.

Stress has been shown to be a significant factor in decreasing the resistance to tuberculosis in experimental models. Tobach and Block (1956) demonstrated that mice, stressed by crowding, and infected with *M. tuberculosis* resisted the disease better if the stress was applied before, but not after inoculation, rather than vice versa. Males also seemed to be more susceptible to the effects of this environmental stressor. This suggests that the hormonal changes induced by the stressor down regulated the immune response to the infection. Activation of the HPA axis in mice was shown to increase the susceptibility of mice *in vivo* to mycobacterial growth (Brown *et al.*, 1993).

Activation of the HPA axis via restraint stress, can increase the *in vivo* growth of mycobacteria in genetically susceptible (*Bcg^f*) mouse genotypes, but does not alter the growth in resistant (*Bcg^r*) animals (Brown *et al.*, 1993). The increase in the susceptibility was directly proportional to the duration of HPA axis activation. This difference did not result from any variation in response to the stress, as the levels of GC produced were equivalent. The response of the macrophages was depressed to the same extent, as measured by levels of anti-mycobacterial factors such as tumour necrosis factor and reactive nitrogen intermediates. It was thought that the increase in susceptibility lay with the ability of macrophages to express MHC class II antigens, and thereby generate appropriate T-helper cell responses, which will commence a cascade of protective CMI responses.

In humans protein-energy malnutrition has been shown to depress CMI responses, including DTH, and LTA responses, although the possibility exists that specific vitamin and mineral deficiencies may also be implicated (Harland and Brown, 1965; Law *et al.*, 1973; Neumann *et al.*, 1975). Protein-energy malnutrition in mice has been shown to mediate some of its detrimental effects via impairment of respiratory burst activity in resident primed macrophages, and reduced phagocytosis and killing of intracellular pathogens (Redmond *et al.*, 1991). In malnourished humans macrophage function is compromised by elevated circulating GC (Hill *et al.*, 1995). Low serum albumin levels may also contribute to increased concentrations of

unbound active GC in individuals suffering protein malnutrition (Neumann *et al.*, 1975). Protein deficiency in guinea pigs has also been shown to produce a reversible loss of T cell reactivity to tuberculin, to impair DTH responses to PPD, and to decrease the efficacy of BCG vaccination (McMurray *et al.*, 1989a). Protein malnutrition also impaired the ability to control the accumulation of viable mycobacteria within lesions, as well as the haematogenous dissemination of bacilli to other organs. These impairments to the cell-mediated functions of protein deficient guinea pigs were also shown to be associated with a deficiency of IL-2, and a hypothesised deficiency of IL-2 receptors (McMurray *et al.*, 1989b). This is consistent with the notion that elevated GC are involved in the impaired CMI response to protein-energy malnutrition, as GC are believed to depress IL-2 production and receptor expression (Lan *et al.*, 1995). In rabbits a deficiency of monocyte derived IL-1 was also shown to be involved in the depression of LTA responses (Bell *et al.*, 1986). Complete and rapid reversal of immune dysfunction in guinea pigs occurs upon restoration of normal nutrition (McMurray, 1994).

In support of the experimental studies on the effect of nutrition on tuberculosis, a case-control study conducted in Ireland, investigating the factors involved in recurrent farm outbreaks of tuberculosis in dairy cattle herds, found that nutritional factors were involved in disease recurrence (Griffin *et al.*, 1993c). Rough grazing on poor quality soils and failure to supplement with mineral licks substantially increased the risk of having a chronic tuberculosis problem. This suggested that not only was protein-energy malnutrition a risk factor for tuberculosis, but that mineral and trace element deficiencies may also be involved.

In possums subjected to the stress of captivity, lymphocyte mitogenic responses are depressed, and a stress leucogram is also found in the initial weeks following capture (Buddle *et al.*, 1992). This suggests that the possums suffer a prolonged stress response associated with confinement. Possums are particularly sensitive to the metabolic actions of GC (Khin Aye Than and McDonald, 1974), which by corollary also suggests that their immune system may be similarly sensitive to elevations of circulating GC. Wild-caught possums will often die in captivity despite the provision of adequate food and shelter (M. Perrott, pers. comm.). Many of the possums appear to pine and waste prior to death and exhibit no lesions at necropsy, but some are affected by stress related stomach ulcers, *Salmonella*

infections and nematode burdens (Keber 1979). Stressed possums in poor condition have also been shown to have higher total and free circulating cortisol levels than animals in better condition, and this was associated with adrenocortical hyperplasia, focal necrosis and haemorrhage (Presidente and Correa, 1981). In an experimental *M. bovis* infection of three possums, Corner and Presidente (1981) found one possum which was showing adrenocortical hyperplasia, focal necrosis and ulceration of the gastric mucosa and ileal villus atrophy, to be the most severely affected animal by the infection. An earlier experimental study also implicated a stress induced immunodeficient state, as a contributor to the rapid development of disease and death in *M. bovis* infected possums (Corner and Presidente, 1980).

Glucocorticoid levels in tuberculosis

In human tuberculosis adrenocortical depression is usually seen, and is believed to be proportional to the severity and chronicity of disease process (Uete, 1962; Beisel and Rapoport, 1969; Srivastava *et al.*, 1980). The pathogenesis of adrenal dysfunction in tuberculous patients has not been clearly established, but destruction of adrenal tissue by the disease process is not thought to be important, as it is uncommon (Clarke *et al.*, 1954; Rybak, 1965), and 80-90% of the adrenal cortex needs to be destroyed before hypoadrenocorticism becomes apparent in cases of tuberculosis (Nichols, 1968).

It was believed at one time that low GC levels were secondary to low ACTH output from the pituitary gland (Uete, 1962; Beisel and Rapoport, 1969). However, a poor GC response to exogenous ACTH, and successful dexamethasone suppression tests in humans with both chronic and newly diagnosed disease, showed that the reduction in circulating GC is actually related to primary adrenocortical insufficiency (Ellis and Tayoub, 1986; Sarma *et al.*, 1990). However, the usual response to acute illness caused by bacterial infection in humans, is for a rise to occur in GC production proportional to the severity of the symptoms (Beisel and Rapoport, 1969). It has been suggested by Brown *et al.* (1993), that chronic inflammatory events, such as tuberculosis, that lead to macrophage activation and the production of IL-1, IL-2, IL-6, IFN- γ , and tumour necrosis factor, may result in increased GC levels, but this has not been substantiated. Elevated levels of GC have been seen in newly diagnosed cases of human tuberculosis (Srivastava *et al.*,

1980; Sarma *et al.*, 1990), but the cause and implications of this observation are unknown.

High GC stress responders are better able to curtail their own bodies' inflammatory responses, but at the expense of compromising cell-mediated immunity. These high stress responses affect cytokine production and MHC class II expression of macrophages such that humoral immunity is stimulated at the expense of protective CMI activity. This may allow intracellular parasites, such as *M. bovis* to establish infection, or allow latent infections to progress. This immune reactivity is underpinned by the resistance genes (Bcg/Nramp) to intracellular parasites, which if present, will be protective of challenge by *M. bovis*, even in the presence of elevated levels of circulating GC.

Species

Red Deer (Cervus elaphus)

Introduction

Although the deer studies reported in this thesis involved red deer only, this review includes comments about other species where they are relevant to the discussion, or are needed to keep observations in perspective. As there is no present evidence which suggests that tuberculosis affects the various species of deer differently, in any major respect, these inclusions seemed warranted.

Ecology

Red deer were first introduced to New Zealand in 1851. By 1919 more than 250 were imported either directly from Britain or indirectly via Australia. By 1923 approximately 1000 red deer had been released into the wild, and by 1940 they had colonised most suitable areas in both the North and South Islands (Challies, 1990). They are currently widespread over most of the South Island, and much of the central and southern North Island.

There are now an estimated 250,000 wild deer in New Zealand. Red deer are the most numerous and widespread, but there are also widespread populations of fallow deer (*Dama dama*), and a large and expanding population of sika (*Cervus nippon*) in the central North Island area. A small population of sambar deer (*Cervus unicolor*) live in the lower reaches of the Rangitikei river. Densities of red deer are generally below 5/km² on the South Island, and between 5 and 10/km² on the North Island.

Carrying capacities of New Zealand forest habitat are likely to be in the vicinity of 20 to 30 deer /km² (Nugent, 1994a). Deer numbers are continually suppressed by hunting activity from the air and ground, the intensity of which is related to the venison price schedule. Thus deer populations are largely confined to sheltered forested country.

Hinds produce a single offspring in early summer each year, commencing at two or three years of age. The majority of calves are weaned in the winter and spring, when approximately 6-8 months old. Deer can live up to 20 years of age, but the average age of deer shot in the wild is between 3 and 5 years. Red deer are sociable animals, forming single-sex groups for most of the year. The female groups consist of adult hinds, their calves and older offspring, with up to 150 animals in a mob. The male groups consist of 2 to 3 year-old stags and older individuals, in mobs of up to 30 animals. Most populations are predominantly female, as the stags are selectively hunted. Female deer usually remain in or near the range occupied by their mothers, but these territories commonly overlap those of other matriarchial groups with which there is likely to be some interaction. Red hinds usually have home ranges of 100 to 200 ha, whereas stags range over an area twice the size. These ranges will increase to thousands of hectares when cover is patchy, availability of food varies, or disturbance levels are high (Nugent, 1993). Males range widely, forming temporary associations with other groups of deer through their lifetimes. Males usually disperse when over 12 months old, but often only travel a few kilometres. However, some have been recorded to move up to 32 km from their birth site (Gibb and Flux, 1973). Hinds disperse over shorter distances, with few moving more than 3 km, and very few moving more than 10 km (Nugent, 1993).

Signs of Tuberculosis

Beatson and Hutton (1981), reported that most farmed deer with tuberculosis in New Zealand die suddenly without premonitory signs, whereas only a few show signs of emaciation or coughing, whilst others develop subcutaneous abscesses which may burst and drain. This varies from the disease in cattle where common pulmonary involvement is characterised by a chronic cough due to bronchopneumonia (Radostits *et al.*, 1994) and draining lesions do not occur. Deer can survive without apparent distress with little functional lung tissue (Quinn and

Towar, 1963), which is in accord with observations in New Zealand whereby deer seem to be in good health whilst suffering from generalised tuberculosis, until the last weeks or days of life (Beatson and Hutton, 1981). The disease process is clearly fulminating in its terminal stages.

Experimental infections (de Lisle *et al.*, 1983; Corrin *et al.*, 1993) have also shown that intratracheally inoculated deer do not usually cough, despite showing signs of respiratory embarrassment or having nodules in the lungs. However, coughing was reported in deer intravenously inoculated with *M. bovis*, but only in those animals showing signs of dyspnoea prior to death (de Lisle *et al.*, 1983).

The apparent difference in susceptibility between cattle and deer, whereby epidemics occur in farmed deer (Beatson *et al.*, 1984; Robinson *et al.*, 1989; Mackintosh and Griffin 1994), may in part stem from the occurrence of suppurating sinuses in deer. These exudates contain multitudes of bacilli, and will be highly infectious to in-contact animals. Likelihood of direct transmission will be enhanced in intensive farming systems as the deer with advanced disease and draining lesions will be victimised, and thus expose their aggressors to a high risk of infection.

Pathology

The most commonly observed lesions in deer are abscesses involving lymph nodes, which may be up to 20cm in diameter and contain up to 3L of pus (Hutton, 1979; Beatson and Hutton, 1981; Fleetwood *et al.*, 1988). Caseous and grossly calcified lymph nodes are rare, with the most commonly found nodal lesion being foci of cream-coloured mucinous exudate. In some cases sinuses open to the skin or other mucosal surfaces will develop when large abscesses form in peripheral lymph nodes. This is likely to occur in the medial retropharyngeal, parotid, caudal cervical and subiliac nodes, but has also been seen when large adherent pulmonary lesions drain through the chest wall and integument (Beatson *et al.*, 1984). The microscopic features of *M. bovis* infections in deer usually include caseous necrosis, mineralisation, epithelioid macrophages, giant cells, and fibrosis (Rhyan and Saari, 1995). The lesions in fallow and red deer/wapiti are very similar, but sika deer show fewer neutrophils, less fibrosis, and many bizarre giant cells (Rhyan and Saari, 1995). Early lesions appear as oedematous/reactive nodes with extreme macrophage accumulation (Kollias *et al.*, 1982). Langhan's giant cells are commonly found but their abundance is not associated with the number of AFB

(Corrin *et al.*, 1993). AFB can be numerous in lesions, but are usually scarce and unable to be found in histological sections or even in smears from purulent lesions (Beatson and Hutton, 1981; Fleetwood *et al.*, 1988). Fewer AFB are found in animals which are resistant to infection, and lesions in these animals are characterised by fibrosis and encapsulation (Corrin *et al.*, 1993). That most deer show some acquired resistance to the disease process is demonstrated by AFB being visible histologically only in lesions from one third of all deer with *M. bovis* isolates, examined at Deer Slaughter Premises (DSPs) in New Zealand (Hathaway *et al.*, 1994).

When the respiratory tract is involved a variety of pathologic changes have been recorded. Discrete nodules from 0.3 to 2.0 cm in diameter, or large abscesses associated with consolidation, and cavitation may develop in the lungs (Clifton-Hadley and Wilesmith, 1991). Granulomatous pleuritis occurs commonly, but pleural effusions occur rarely and have only been reported once in a hog deer (*Axis porcinus*) (Basak *et al.*, 1975). Miliary lesions of the lungs may also be present, especially in deer calves (Philip, 1989). The respiratory lymph nodes often enlarge and abscessate.

Tuberculous lesions also occur in joints, spleen, liver, ovaries and eyes of advanced cases (Hutton, 1979). Lesions have been found in the oropharyngeal tonsil by several investigators. Lesions ascribed to tuberculosis in tonsils were reported in 5 of 187 deer examined by Beatson *et al.* (1984), in a herd with a high prevalence of tuberculosis. Three of 73 tuberculous deer were also reported to have tonsillar lesions consistent with tuberculosis in a North American study (Rohonczy *et al.*, 1996). Brooks (1984) also describes the isolation of *M. bovis* from lesions in the tonsils of 3 of 36 intratracheally inoculated deer and 3 of 15 in-contact controls. These lesions were described as focal, pea sized, cream coloured nodules, indistinguishable from other caseous lesions caused by trapped crypt debris. A purulent tonsillitis was found in one of six deer examined by Fleetwood *et al.* (1988) but the lesion was apparently pooled with other tissues for *M. bovis* isolation, and so it was impossible to determine the cause of the lesion. Mackintosh and Griffin (1994) also found lesions in tonsils after experimental tonsillar crypt inoculation, but noted that lesion development at this site is uncommon.

Lesion distribution

In an analysis of lesion distribution in 668 tuberculous farmed deer, inspected at slaughterhouses in New Zealand between 1990 and 1993, Hathaway *et al.* (1994) found that 74.7% had single lesions, while 9.6% had more than two lesions. Over fifty two percent of lesions were in the retropharyngeal lymph nodes, while 20.8% were in the ileo-jejunal lymph nodes, the next most common single site. Respiratory lymph nodes were involved in 23.8% of cases. In 8.5% of animals lesions were on the pleura, and in 8.1% lesions were in the lungs. This lesion distribution is similar to other reported studies (Beatson, 1985; Beatson *et al.*, 1984; Wilcockson, 1986; Mackintosh and Griffin, 1994; Rohonczy *et al.*, 1996).

The predominance of lesions in the head differs from the lesion distribution in cattle where the majority are associated with the respiratory tract both overseas (Francis, 1958; Neill *et al.*, 1994) and in New Zealand. From 1398 lesioned reactors killed between 1986 and 1988 in the Taumaranui district of New Zealand, Crews (1991) reported that 64.7% had respiratory tract lesions, and 52.9% had thoracic lesions only. The medial retropharyngeal lymph node was the next most frequently affected site (17.9% of cases). These data suggest that the majority of transmission to cattle occurs by the respiratory route, whereas in deer the principal route of infection may be by the alimentary tract, particularly via the tonsillar lymphoepithelium.

Evidence suggests that in some cases infection will be present without development of grossly visible lesions, or with resolved gross lesions. Beatson (1984) found 25/107 (23%) of positive skin test reactor deer without gross lesions to be infected, after separate pools of lymph nodes collected from the head, thorax, abdomen and body were cultured. Philip (1989) reported that 11/32 (34.4%) culture positive deer from a farm outbreak in Northamptonshire showed no gross lesions at necropsy. Seven of 26 (27%) culture positive imported red deer in England were also found to have no gross lesions when necropsied (Stuart *et al.*, 1988). Similarly, Griffin *et al.* (1991) found lymphocyte transformation test evidence of infection in 9 of 48 (19%) deer which showed no gross lesions.

Pathogenesis and transmission

The infecting dose of organisms has long been recognised as a determinant of the subsequent course of the disease. It has been hypothesised that large doses of *M.*

bovis, as well as overwhelming local phagocytic defence mechanisms, will lead to the establishment of the T-helper 2 cellular response which eventually leads to proliferation of non-protective humoral immune activity (Mossman and Coffman, 1987; Griffin *et al.*, 1993b; Baird *et al.*, 1995). Lower infecting doses are more likely to lead to protective antigen-presenting cell cytokine responses, and effective antigen presentation to T-helper cell clones. This leads to a protective T-helper 1 cell response which in turn promotes the development of acquired resistance, without necessarily developing delayed-type hypersensitivity (DTH), and to containment or elimination of the disease process. Thus where infected deer excrete few organisms there is less likelihood of disease establishment in cohorts, whereas if an in-contact deer receives a large dose of organisms, even a moderately resistant animal may succumb to the disease process. As few as eight bacilli inoculated into the tonsillar crypts have been shown to cause widespread disease in susceptible individuals (Mackintosh and Griffin, 1994).

Severe outbreaks in deer and high rates of transmission have occurred only where there has been advanced disease of the respiratory tract, or suppurating sinuses in some animals. In the most outstanding outbreak reported (Robinson *et al.*, 1989) 92% of 51 fallow deer on three farms were infected. Ten animals were identified with draining skin lesions. Another disease outbreak where 39.8% of 337 wapiti were infected, may have been exacerbated by one animal with a retropharyngeal node lesion draining to the pharyngeal mucosa (Whiting and Tessaro, 1994). One wild-caught farmed deer with a draining skin abscess was identified early in an outbreak reported by Beatson *et al.* (1984), in which 94 animals with tuberculous lesions were identified in a herd of several hundred red deer. In another outbreak near Invermay, Mackintosh and Griffin (1994), reported three with draining skin lesions, where 123 of 250 (49%) deer developed lesions. In a well managed accredited tuberculosis free herd in the Hawkes Bay area (Atkinson, 1993), rapid spread within the herd was associated with a draining mandibular abscess in one hind. Lesions were seen in 81 of 503 (16.1%) deer. Of 170 weaners skin tested, eight of 12 test positive were identified with lesions, six of those were from the mob in which the hind with the draining mandibular lesions was present. Even though highly infectious individuals were present, the transmission to the calves appeared to be minimal. In a trial reported by Brooks (1984) and Carter *et al.* (1984) where 36 red deer were intratracheally inoculated with 1 μ g of *M. bovis*, 12 of 15 (80%)

non-inoculated in-contact control deer became infected and diseased over a period of 48 weeks. This prevalence is likely to have resulted from the severe pulmonary lesions present in many of the inoculated animals and the extended period of contact.

Other experimental studies have not reported high rates of infection in in-contact control deer. Mackintosh and Griffin (1994) describe infection (retropharyngeal) in only one of 20 in-contact controls, after 8 months with a group of 30 inoculated animals, some of which had severe pulmonary lesions and were subject to frequent close contact through yarding and supplementary feeding. In two other 8-month trials, one of eight controls was infected while in-contact with 24 intratonsillar-inoculated animals, and none of eight stags were infected when run with 22 intratonsillar-inoculated stags, despite severe generalised disease being found in four and one of the inoculated deer in each experimental group respectively. In another study none of four in-contact, un-inoculated stags became infected whilst being run with 34 other infected stags, despite many deer having generalised disease (Mackintosh *et al.*, 1995). No draining skin lesions were reported in these trials.

Paterson (1993) describes the more typical, but usually unreported, type of outbreak of tuberculosis in a deer herd in which the prevalence remains low. This outbreak occurred in a fallow deer herd near Rotorua, in which only a single mob of mixed age hinds were infected. Twenty two of 356 (6.2%) were found to have lesions, but none were generalised or severe. There had been no apparent transmission to offspring weaned some 4 to 6 months earlier and no evidence of spread across fence lines to other mobs of cattle or deer.

Routes of infection

Explanation for transmission of tuberculosis from deer to deer or from other animals to deer must address the high prevalence of lesions in the head (and in particular the medial retropharyngeal lymph node) found in infected deer in New Zealand. Several attempts to establish an experimental model which mimics the natural process of infection in deer are described below.

Intravenous inoculation of three red deer (de Lisle *et al.*, 1983) produced miliary lesions in the lung, and at the highest dose only ($>100\mu\text{g}$) was there histological evidence of disease in the kidney, spleen and liver. All three deer died of

generalised tuberculosis within 28 days of inoculation. The susceptibility of the lung to haematogenous infection in deer may be due to the presence of pulmonary intravascular macrophages (Carrasco *et al.*, 1996), which outnumber pulmonary alveolar macrophages by two to one, and have been shown in other ruminants to clear the blood of the largest proportion of blood borne bacilli (Winkler, 1988). The absence of lesions reported in the liver, spleen and kidney and other potentially haematogenously infected sites indicates that bacillary survival must be severely curtailed in these organ-associated compartments of the immune system.

Subcutaneous inoculation of four deer (de Lisle *et al.*, 1983) produced local skin abscessation and local caseous lymph node lesions, with signs of systemic spread to the thorax in only one animal in which the dose was higher than 100 µg.

Intratracheal inoculation of three deer with doses from 1 µg to 100 µg produced gross lesions in the cranial lung lobes and the bronchial lymph nodes only, with no sign of spread to other tissues examined histologically (de Lisle *et al.*, 1983). In another trial (Brooks, 1984), 36 deer were intratracheally inoculated with 1.0 µg *M. bovis*, with 32 deer developing tuberculous lesions. Thoracic organs contained 57% of lesions seen, but a significant proportion were also found in the head (15%) and abdomen (26.5%). No thoracic lesions were found in 5 of the 36 inoculated animals, suggesting that the lung is resistant to lesion development in some deer, or that the inoculum was brought up by the bronchial mucociliary elevator before infecting alveolar macrophages or pneumocytes, and bacilli were thus available for entrapment by the oral or gut mucosa only. Haematogenous dissemination, to the carpus and eye, was found in 10% of the animals, suggesting that dissemination via the bloodstream was common.

In another trial in which 60 red deer were intratracheally inoculated with 1 µg of *M. bovis* (Corrin *et al.*, 1993) fifty three (88%) of the deer developed thoracic lesions, which led to the death or decision for euthanasia of 34 animals within 4 months of inoculation. Mackintosh *et al.* (1993) intratracheally inoculated 2 groups of 5 red deer with either 10^2 or 10^4 *M. bovis* organisms, resulting in moderate to severe thoracic lesions in seven, causing the death of one prior to necropsy at 35 weeks post inoculation.

Intranasal instillation of 4 ml of coarse spray containing either 10^2 or 10^4 *M. bovis* organisms was carried out in two groups of five deer by Mackintosh *et al.* (1993). The low dose resulted in only one developing a gross lesion in a mediastinal lymph node. All five of the high dose group developed lesions, including three with extensive head node and thoracic involvement, and one each of retropharyngeal and mediastinal node, and retropharyngeal and ileocaecal lymph node involvement (Mackintosh and Griffin, 1994).

Intratonsillar inoculation, by instilling either 10^2 or 10^4 *M. bovis* organisms into a tonsillar crypt of two groups of five red deer, was reported by Mackintosh *et al.* (1993). The resulting lesions were confined to the draining medial retropharyngeal lymph node in four of both the low and high dose animals. One of the high dose animals also had an associated mesenteric node lesion and one with no gross lesions had an infected tonsil (Mackintosh and Griffin, 1994). Further studies by Mackintosh *et al.*, (1995), involving intratonsillar inoculation of 5×10^2 organisms established infection in 34 of 39 stags. All 30 diseased animals, had head-associated lymph node lesions, and 21 of those deer had lesions in other sites. Mackintosh *et al.* (1995) reported that deer can be experimentally infected with as few as eight *M. bovis* colony forming units (cfu) when inoculated into the tonsillar crypts, resulting in gross lesions in 50% of the animals. When the dose was increased to 200 - 500 cfu, 70 to 100% of the deer became infected showing more severe pathology than those infected with a lower dose.

The above observations show only intratonsillar inoculations have produced a pattern of disease similar to that of natural infection. This suggests that the most common route of infection in deer may be by the oral route, with the bacilli initially taken up by the oropharyngeal tonsil. Lymphatic drainage from the caudal nasal and pharyngeal mucosa, including the oropharyngeal tonsils, passes directly to the medial retropharyngeal lymph nodes (Barrel and Simpson-Morgan, 1990; Nickel *et al.*, 1981). This drainage pattern is likely to explain the common occurrence of lesions at this site subsequent to oral contamination with bacilli. Infection may thus occur during a variety of normal activities such as eating, drinking, licking, biting, muzzling and grooming, and inquisitive behaviour towards wildlife, as described by Sauter and Morris (1995a).

Mackintosh *et al.* (1995) has shown that natural transmission from tonsillar inoculated deer to in-contact non-infected deer occurs at only a slow rate (<10%) in the first 6 months, under field conditions, despite a number of infected deer having lesions in the tonsils and thorax. Montgomery, (1987) also noted that tuberculosis spread in a number of herds where the only lesions found were in the retropharyngeal lymph nodes. This implies that there may have been shedding of bacilli from a non-lesioned site.

Genetic susceptibility

Innate resistance in deer, as proposed by Griffin *et al.*, (1993a), probably plays an important role in protecting some deer from infection. This natural resistance may be controlled by a single dominant gene, Bcg/Nramp, the presence of which has been demonstrated in mice and hypothesised for cattle (Frelief *et al.*, 1990; Qureshi *et al.*, 1996). This gene has been shown to confer resistance to intracellular bacteria, such as *M. bovis* and *Brucella abortus*, through its effect on intracellular killing of bacteria in phagolysosomes. The Bcg/Nramp equivalent gene from deer has recently been cloned from deer monocytes, and breeding trials are underway at Invermay to assess its role in the resistance to *M. bovis* infection (Mackintosh *et al.*, 1995).

Acquired resistance is also likely to be important with deer, and this occurs in animals which mount a successful immune response to a challenge with *M. bovis* infection. The major histocompatibility complex glycoproteins (MHC) control the development of specific acquired immunocompetence, and hence are critical to development of effective acquired resistance. Strong evidence for either acquired, or innate resistance providing protection for deer has been gained in the course of experimental studies in which deer have been inoculated with viable *M. bovis* and subsequently been found uninfected at necropsy. Corrin *et al.* (1993) inoculated 60 deer with 0.1 µg of *M. bovis* intratracheally. One hundred and twenty days post-inoculation, two which survived appeared to be free of infection, and one other culture positive deer showed no gross lesions. Mackintosh *et al.* (1995) observed four of 39 inoculated deer having no lesions after six months, but with infection in the tonsils. A further five deer had no evidence of infection whatsoever. Griffin *et al.* (1988a) showed approximately 12% of in-contact deer may show transient rises in LTA reactivity, which has been interpreted by these workers as indicative of

exposure to *M. bovis*, and acquisition of immunity without disease development (Buchan *et al.*, 1990).

Stress and glucocorticoids

A study by Thomson *et al.* (1994) showed that chronic low-level administration of dexamethasone, in an attempt to mimic chronic stress, increased the number of infected red deer and the severity of disease after challenge with *M. bovis*. Prolonged release of glucocorticoids increases the susceptibility to a wide range of pathogens by exerting a profoundly suppressive effect on the cell-mediated immune response. It has been hypothesised that the florid presentation of tuberculosis infection in deer herds throughout the 1980s, may have been related to the stress of recent introduction of wild deer into captivity, and the limitations of the crude management practices prevailing at the time of industry establishment (Thomson and Griffin, 1995).

Management stress and overcrowding have jointly been implicated in some of the most severe outbreaks of tuberculosis reported in deer. A prime example (Robinson *et al.* 1989), involved three herds of fallow deer in Australia, where overstocking and environmental stressors appeared to play a role in the development and transmission of tuberculosis in an unusually severe outbreak of disease, where 92% of 51 deer developed disease. An 89% prevalence of tuberculosis was also reported in a group of 96 deer of mixed species gathered together from wildlife parks, and held in 0.5 to 1 acre pens for tuberculosis test evaluations (Kollias *et al.*, 1982). Overcrowding and social stresses were likely to have contributed to the high prevalence recorded.

Age effects

There is a small but consistent body of evidence which suggests that the disease in deer less than 6 months of age differs in important respects from that seen in older animals. The experience of the Deer Tuberculosis Diagnostic Laboratory at Dunedin (Griffin and Buchan, 1994), is that animals “under 6 months of age, if infected with *M. bovis*, will not show pathologic and immunological responses characteristic of tuberculosis in adult animals. Young animals with no obvious macroscopic lesions may harbour large numbers of *M. bovis* organisms within their lymphatic tissues”. Experimental observations in England suggest that under certain conditions fawns are more susceptible to infection and may suffer a more

acute course of disease than adults (Anon, 1990). This may explain why Robinson (1989) noted that the worst affected deer in the outbreak he reported was a 4 month-old doe, which had the largest abscesses and most widespread disease. The observation of heavy bacillary loads in the lymph nodes of young deer, in the absence of gross lesions may be due to the immaturity of the immune system, which early in neonatal life may be more concerned with expressing tolerance for self antigens rather than developing functional CMI to all antigenic stimulation. The activity of suppressor T-lymphocytes is enhanced, antigen-presenting cells function poorly and inflammatory responses are of lower magnitude in neonates (Watson and Gill, 1991). Thus *M. bovis* may gain entry to antigen-presenting cells, which subsequently fail to process and present bacillary antigens to lymphocytes, thus allowing a period of unrestricted multiplication (Yu *et al.*, 1979), without the significant host response normally orchestrated by macrophages (Cross *et al.*, 1996). The delay in the usual DTH inflammatory response ensures that the development of lesions is retarded, but once the response is initiated, the ensuing disease may be more severe than is commonly seen in older animals.

Although once infected, the development of disease in young deer may be more severe than adults, the prevalence of disease seems to be lower than for older animals (Atkinson, 1993; Paterson, 1993; Whiting and Tessaro, 1994). There have been no reports of congenital tuberculosis in deer, whereas in herds of cattle with a high prevalence of disease, up to 0.5% of calves may be infected via the umbilical vessels (Jubb *et al.*, 1993). The spread of disease by ingestion of milk from infected mammary glands has also not been reported in deer. However Fletcher (1990) found infection without gross lesions in three 6 month-old deer calves taken from infected mothers at birth, suggesting that at least one was infected by its mother. The prevalence of lesions (6%) in wapiti calves in the abattoir study of Whiting and Tessaro (1994), was significantly lower than other classes of stock examined in a depopulated herd of 337, which had a disease prevalence of 40%. Griffin (1988) also found no evidence of disease in 80 six month-old calves which had been running with adult hinds, in a herd of deer with up to a 90% prevalence in mobs of adult stock.

It appears as though neonatal deer are susceptible to infection with *M. bovis*, but that visible expression of disease may be initially limited by the immaturity of the

immune response. However, there may be some behavioural characteristics operating which reduce the risk of deer calves becoming infected, despite having a close association with infected dams.

Routes of excretion

One hundred and eighty five nasal swabs, regularly taken from 36 intratracheally inoculated deer, were subjected to mycobacterial culture by Corrin *et al.* (1987). No isolates of *M. bovis* were recovered from the samples, despite 32 of the deer showing lesions at necropsy, and three of the animals dying from tuberculosis. The findings indicated that *M. bovis* was not present in large numbers in the nasal cavities of known infected deer. Although nasal swabs and bronchial lavage fluid were collected and cultured from a number of experimentally infected deer by Mackintosh *et al.*, (1993), no results of this culturing have yet been reported. It is therefore not known whether organisms can be cultured from the respiratory tract or other sites from infected deer. There have been no other records of excretion site sampling conducted on deer, and more research is clearly required.

International situation

Tuberculosis has been observed and reported in wild cervids in a number of countries around the world, often in association with tuberculosis in other species (see Table 2-I). The following reports examine the relationship between other species as sources of *M. bovis*, and the infection in deer, with the aim of clarifying whether wild deer can maintain the disease amongst themselves, without introduction from outside sources.

North America

In Canada *M. bovis* has been observed in wild cervids ranging in the same area as a heavily infected herd of bison (*Bison bison*) at Buffalo Park, near Wainwright, Alberta, in the 1930s (Hawden, 1942). Lesions due to *M. bovis* were seen in 73 (5.5%) of 1329 wapiti (*Cervus elaphus nelsoni*), six (5.6%) of 107 moose (*Alces alces*) and two (0.8%) of 242 mule (*Odocoileus hemionus*) deer which ranged in the same area, but not in close association with the bison. It was suggested that the tuberculosis prevalence in the wapiti was higher than in the mule deer because of their habit of running as a mob, and possibly their different grazing habits.

Table 2-I. Prevalence of infection and disease caused by *M. bovis* in free ranging deer populations (adapted from Clifton-Hadley and Wilesmith, 1991)

Species	Prevalence	Origin	Comments
Wild deer			
Fallow, red, sika	5/130 (3.8%)	Republic of Ireland (Dodd, 1984)	Study of deer killed by hunters in area with cattle breakdowns and infected badgers
Sika, roe	5/117 (4.3%)	England (Rose, 1987)	Investigation of cattle breakdowns
Sika, roe	3/232 (1.3%)	Great Britain (Anon. 1989)	Two from Dorset associated with infected badgers. One roe deer from Invernesshire
Unspecified	8/734 (1.1%)	England (Philip, 1989)	Cases associated with cattle breakdowns and infected badgers
Red	(0.09%)	Germany (Witte, 1940)	Based on gross pathology
Axis	(<5.0%)	Hawaii (Essey <i>et al.</i> , 1981)	Still known to be present but no cases identified
Roe	11/892 (1.2%)	Switzerland (Bouvier, 1963)	Associated with infected cattle
White-tailed	1/440 (0.2%)	USA (Belli, 1962)	Survey
White-tailed	(>4.0%)	USA (Schmitt, 1995; B. Corso, pers. comm.)	“Managed” wild deer, prevalence based on hunter survey
Mule	2/41 (4.9%)	USA (Rhyan <i>et al.</i> , 1995)	Associated with infected wapiti on adjacent game ranch
Red	16/72 (22.2%)	New Zealand (Fraser <i>et al.</i> 1994)	Associated with infected possums and cattle outbreaks
Red, sika	10/55 (18.2%)	New Zealand (Nugent and Proffitt, 1994)	Associated with infected possums and cattle outbreaks
Red	1/48 (2.0%)	New Zealand (Nugent and Mackereth, 1996)	Associated with cattle outbreak only
Park Deer			
Wapiti	73/1329(5.5%)	Canada (Hawden, 1942;	Ranging with a very heavily infected bison herd
Moose	6/107(5.6%)	Tessaro, 1986)	
Mule	2/242(0.8%)		
Unspecified	1/460 (0.2%)	England (Fletcher, 1991)	Lightly stocked free ranging deer

Two tuberculous white-tailed deer (*Odocoileus virginianus*) were found in New York state earlier this century (Levine, 1934), from counties where a high prevalence of tuberculosis in dairy cattle existed. It was thought that mingling with the cattle at pasture, may have been the cause of disease in the wild deer. Belli (1962) reported one case of parietal pleural tuberculosis in a group of 440 white-tailed deer carcasses examined in Ontario. No information was given on the likely exposure of this animal to tuberculous cattle herds, but the possibility of infection being maintained in the absence of infected cattle was raised.

Since 1989, tuberculosis has been detected in captive deer herds in eight states in the USA, and in three Canadian provinces, attributed to initiation by contact with infected bison, and disseminated through subsequent animal dispersal by sale (Essey and Koller, 1994). Surveillance and control are currently underway in both countries. Tuberculosis has recently been diagnosed in 2/41 (4.9%) wild mule deer on a cattle ranch in Montana adjacent to an infected game farm, in which 12/150 (8%) wapiti were infected with *M. bovis* (Rhyan *et al.*, 1995). No likely source of the infection for the captive wapiti was given, so it could not be determined from the report whether the wild deer were infected by, or transmitted the infection to, the game farm wapiti. Five cases of farmed deer-cattle transmission of *M. bovis* were recorded in the USA in 1991-1992 (Bleem *et al.*, 1993, cited in O'Reilly and Daborn, 1995).

During 1995, tuberculosis was identified in managed "free-ranging" white-tailed deer in northern Michigan (Schmitt, 1995), associated with the transmission of infection to one of approximately 1000 cattle in the area (B. Corso, pers. comm.). The prevalence in the deer has been estimated at approximately 4%, from information provided by hunters and other limited investigations. The true prevalence is probably substantially higher, as hunters have been shown to recognise only about 10% of infected deer (Nugent, 1994b). This high density deer population is hand fed and even yarded during the winter. This is the only report of tuberculosis in deer in the USA in which cattle to deer transmission cannot explain the findings, as there have been no tuberculous cattle in the area for many years. Tuberculosis had earlier been found in white-tailed deer from Michigan by Ferris *et al.* (1961), who reported two cases of tuberculosis in deer which had come from an undetermined location in the early 1960s. Another infected deer was found in the

same area as the current outbreak, in the 1970s, at which time it was assumed that tuberculosis would not be self-maintaining in wild deer populations, and that the infection was related to contact with tuberculous cattle, which were still present at that time.

Hawaii

Wild axis deer (*Axis axis*) on the Hawaiian island of Molokai were also infected with *M. bovis* (Sawa *et al.*, 1974; Essey *et al.*, 1981). These deer were known to mingle with domestic and wild cattle, both of which were also infected with tuberculosis. A 20% prevalence of tuberculosis in wild pigs was also found on the island. However, Essey *et al.* (1981) found no evidence of infection in 100 wild deer necropsied, despite tuberculosis still being known to be present in the deer population. From this study it was estimated that the prevalence in the deer was low, below 5%. It is now thought that the disease has been eradicated from the deer, subsequent to the destocking of cattle farms (Nugent and Proffitt, 1994).

Britain

Isolations of *M. bovis* were first reported from wild deer in Britain in 1981 (Matthews *et al.*, 1981). Two isolated cases of tuberculosis caused by *M. bovis* in wild roe deer from north Wiltshire, in 1980 and in 1984, raised the possibility of the presence of a wildlife reservoir of infection in this area in which badger (*Meles meles*) tuberculosis is endemic (Gunning, 1985). The infected wild deer in Wiltshire and those subsequently found in Dorset apparently are restricted to geographically well defined areas and have a tuberculosis prevalence of 1.6% (MAFF reports, 1985 and 1986). However, Rose (1987) later reported 5/117 (4.3%) sika and roe deer infected with tuberculosis in the Purbecks area of Dorset. This focus of infection was associated with tuberculosis breakdowns in 3 cattle herds, and an earlier focus of infection in badgers (which had apparently been eliminated some years previously). Of 734 wild deer sampled from south-west England up to 1989, only eight (1.1%) have been found infected with *M. bovis*, whereas only one of approximately 2000 sampled from south-east England, an area free of tuberculosis in badgers, has been found infected (Philip, 1989). However, in 1989 a further three of 232 wild deer were found infected with tuberculosis. *Mycobacterium bovis* was isolated from one roe (*Capreolus capreolus*), and one

sika deer from Dorset, a county with endemic tuberculosis in badgers, and from one roe deer from Invernesshire, an area free of endemic badger tuberculosis (Anon., 1990). It is somewhat puzzling that the infected roe deer near Inverness, and the other infected deer from the south-east of England have been mentioned only once, and with no reference made as to the likely source of infection, as these animals were from areas without apparent badger infection. It is also known that several lightly stocked deer parks, containing both red and fallow deer subject to extensive grazing management, harbour *M. bovis* infection at low prevalence (Fletcher, 1990 and 1993), and it is believed that the deer herds may have been infected for a prolonged period. In one park established 300 years ago, one of 460 culls (0.2%) has shown evidence of tuberculosis.

Ireland

Infected sika and sika-red hybrids have been found in County Wicklow, Ireland, an area with endemic badger tuberculosis. Five of 130 (3.8%) hunter-shot animals (or portions thereof) were identified with gross lesions containing *M. bovis* (Dodd, 1984). An earlier fatal case of tuberculosis in a wild fallow deer was also reported in the same county in 1976 (Wilson and Harrington, 1976).

Continental Europe

Occasional cases of tuberculosis in roe and red deer were noted in Germany through the 1930s, before the eradication of tuberculosis from the cattle herds (Rankin and McDiarmid, 1968). Vöhringer (1964) reported on the possible reinfection of several cattle herds, twice from roe deer and once from a red deer calf, either through direct contact, or pasture contamination, when the herds grazed near forests.

A Swiss account by Bouvier (1960) reported *M. bovis* infection in 11 of 892 (1.23%) roe deer and two cases in red deer, with extensive lung lesions and large numbers of AFB. Deer were thought to have contaminated pastures and caused reinfection of cattle herds in some areas of Switzerland, and were also thought to have been the cause of tuberculosis in badgers, which was first reported in Switzerland (Bouvier, 1960). However, Pastoret *et al.* (1988) commented that after eradication of tuberculosis from Swiss cattle herds that the wild deer populations also apparently became free of the disease.

New Zealand situation

Tuberculosis was first noted in wild red deer on the West Coast of the South Island in 1956 (Livingstone, 1994), although the Central Veterinary Laboratory did not confirm the presence of *M. bovis* till 1970, in a wild yearling red stag, originating from near Inangahua (Beatson, 1985). The disease is thought to have been present in the Hauhungaroa Ranges for several decades, and may have been spreading slowly northwards during the 1970s and 1980s (Nugent, 1994b). It is possible that tuberculosis was present in the first deer introduced to New Zealand as these were derived principally from British deer parks, some of which have since been shown to be infected with tuberculosis. However the current prevalence of tuberculosis in wild deer is more likely to be due to interactions with tuberculous cattle or wildlife in more recent times.

Before the mid 1970s few tuberculous wild deer had been identified. No cases had been identified at Game Packing Houses (GPH), despite over 100,000 carcasses being processed each year (de Lisle and Havill, 1985). At this stage viscera were not brought in with the animal. In 1975 a change of procedure requiring the inspection of lungs, heart, liver and kidneys, in addition to the carcass, improved the sensitivity of inspection. Submission of many tuberculous cervine lesions to the Central Veterinary Laboratory for culture resulted. In 1980, 62 isolates of *M. bovis* came from GPH submissions (de Lisle and Havill, 1985). The prevalence recorded at GPHs in the early 1980s was less than 0.21% (Hennessy *et al.*, 1986). De Lisle and Havill (1985) reported isolation of *M. bovis* from 161 wild deer, 141 of which came from GPHs, prior to 1985. Most infected wild deer came from the Wairarapa, Central North Island endemic area and the West Coast, but a few came from Fiordland, Otago and Southland (Mackintosh and Beatson, 1985). The majority of isolates were from red deer, but *M. bovis* has been isolated from two wild sika deer and two wild fallow deer (de Lisle and Havill, 1985).

The distribution of lesions in wild deer described by de Lisle and Havill (1985), differs markedly from farmed deer, in that 75% of cases had thoracic lesions. However, this data should not be interpreted as suggestive of a true difference in lesion distribution between wild and farmed deer, as wild deer at that time, had only the thoracic viscera examined, thereby missing any lesions which may have been present in the abdomen or around the head. Inadequate inspection procedures, and

prior culling of grossly affected carcasses by hunters, will have ensured that GPH data will have substantially underestimated the true prevalence of tuberculosis in the source populations. In March 1995, regulations requiring heads be attached to deer carcasses at inspection were introduced, thereby allowing a more accurate determination of the prevalence of gross lesions in wild deer. Since this change in requirements, there has been a three fold increase, to 30%, in the proportion of wild deer from the central North Island endemic area found to be tuberculous (McCabe pers. comm.).

Prevalence of tuberculosis

The prevalence of tuberculosis in wild deer has only been studied in detail within the central North Island endemic area where both sika and red deer are widespread. In the Hauhungaroa Ranges, 39 red deer carcasses were examined between 1989 and 1993, and 7 (18%) were found tuberculous (Nugent, 1994a). In December 1993, 20/33 deer were partially inspected at a DSP, and two were found with lesions. The other 13 were fully inspected in the field and 7 were found with lesions. The observed prevalence of disease found in this group was 27%, but the true prevalence is likely to be higher since head and abdominal contents were not inspected at the DSP.

East of Turangi on the North Island, there has been a focus of infection in deer centred upon the Rangipo and Hautu prison farms, recognised for over 20 years (Nugent and Proffitt, 1994). This is an area of high deer density, with many animals harvested from the area presented to the GPH in poor condition, because of low forage availability. Ten of 55 red and sika deer (18.2%) from this area, which were examined in 1993/94 had tuberculous lesions (Nugent and Proffitt, 1994).

Relationships with other infected wildlife

A MAF survey in 1982/83 conducted on the perimeter of the forested area of the Hauhungaroa Ranges, found a 1.25% prevalence of tuberculosis in 6083 captured possums (Pfeiffer *et al.*, 1995). Other studies in the same area between 1989 and 1995 have found the prevalence of tuberculosis in possums to vary between 0.4 and 4.7% (Nugent, 1994b; Fraser *et al.*, 1995). Although the prevalence of disease in the possums varies, the Hauhungaroa Ranges appear to harbour populations of both deer and possums with a substantial prevalence of tuberculosis at all times.

The 10 tuberculous deer found near the Rangipo and Hautu prison farms, were all from the 10,000 ha endemically infected area of native forest (Nugent and Proffitt, 1994). Of the 55 deer examined, 41% of the 17 taken within 4 km of the forest-pasture margin were infected, whereas only 12% of the 24 deer examined from 4 - 8 km into the forest were infected, and none of 14 taken further than 8 km from the forest-pasture margin showed evidence of disease. A recent hunter survey, described in the same report, found that hunters had identified suspicious lesions in deer taken 5 km to the north east and 10 km to the south of the recognised endemic area. Nugent and Proffitt (1994) believed that if these hunters' reports represent actual tuberculosis cases in deer, that the diameter of the endemic area had spread by less than 1 km per year since the area was first found to harbour tuberculous wildlife. Cross-sectional studies, examining possums for the presence of tuberculosis, have shown that tuberculosis infection in the deer appears to have a spatial association with the presence of infected possums in the Rangipo/Hautu area (Nugent and Proffitt, 1994).

At Timahanga station, in the northern Rangitikei, a tuberculosis free area, one infected wild red deer calf with a single small hepatic node lesion, was found amongst 128 sampled in the vicinity of a new outbreak of tuberculosis in cattle (Nugent and Mackereth, 1996). The infected animal was a 5 month old female, born since the last of the infected cattle were removed from the area. She was the only tuberculous animal found in a group of 48 shot from one mob of deer which were thought to have potential contact with the mob of cattle from in which the reactors came. None of 3,999 possums killed and examined in the area showed evidence of infection, nor did any other species, including six pigs examined. It was thought that tuberculosis was present at very low prevalence in the deer population, and may have been first introduced via escapees from a local deer farm, newly established in the early 1980s.

In a similar incident, one of approximately 50 wild deer was found infected near a recent outbreak in cattle near Waikaka (G. Nugent pers. comm.). In both these instances many thousands of possums examined failed to show evidence of tuberculosis infection, thus raising the possibility that the wild deer may have been the source of infection for the cattle herds, and that in the absence of tuberculous possums the prevalence of infection in the deer is quite low.

A low prevalence of tuberculosis has been found in infected wild deer in a low density red deer population in the Otago region (Nugent, 1994a). This suggests that the disease may persist in low density deer populations and/or that the deer are being continually challenged by new sources of infection e.g. ferrets and possums.

Transmission of tuberculosis from deer to wildlife

It has been speculated that the wild deer of New Zealand may have been responsible for the initial introduction and establishment of tuberculosis in possum populations in what are now regarded as endemic areas (Morris and Pfeiffer, 1995). This hypothesis can never be proven, and relies upon the assumption that the disease was first endemic in the wild deer populations. However, the idea has been given substantial support by two instances where farmed deer appear to have been responsible for the introduction of tuberculosis to possum populations.

Wild deer introduced from the West Coast to Mackenzie Basin farms in the early 1980s have been strongly implicated in the establishment of tuberculosis in possums, ferrets and feral cats of that region (de Lisle *et al.*, 1995). Many of the cattle tuberculosis breakdowns and associated wildlife disease in the Mackenzie Basin were caused by closely related *M. bovis* REA types, which were similar to, or the same as, those found on the West Coast.

Mackereth, (1993) also described how infected deer introduced from the Mackenzie Basin to the Hawke's Bay area, were subsequently implicated in the transmission of tuberculosis to local possum populations. Deer on the index property were thought to be first infected in 1986, and by 1993, 17 cattle and deer herds within a 4 km radius of the initial infected property had experienced breakdowns. All *M. bovis* REA types isolated from stock and possums in the area were of a known Mackenzie Basin isolate.

Farmed deer are known to behave with interest and aggression towards small mammals inside their enclosures, and it is possible that wild deer may behave similarly. It is not uncommon in North America for wapiti and red deer to be found with porcupine quills embedded in their muzzles after molesting these animals (Haigh and Judson, 1993). There have been anecdotal reports of deer in New Zealand running down and killing rabbits by striking them with their forelegs. Thus it is conceivable that a tuberculous deer could be capable of running down and

molesting a healthy possum or other small mammal. If the animal escapes and develops tuberculosis it would have the potential to establish a focus of endemic tuberculosis. Infection may also move in the opposite direction, from possums to deer. Sauter and Morris (1995a) demonstrated that possums and ferrets, sedated to appear like moribund tuberculous animals, not only attract the attention of deer but are often vigorously investigated by sniffing, licking, biting and striking. This kind of aggressive interspecific interaction thus has the potential to transmit tuberculosis in either direction depending upon which of the species is initially infected.

Deer to cattle transmission

Probability of farmed deer infecting cattle appears to be very low, as there have been only four incidents where there is evidence to support its occurrence (Hennessy *et al.*, 1986). Transmission apparently occurred in three instances of cattle grazing behind deer and in one case of infection across a fence. All four situations were associated with a very high prevalence of tuberculosis in the affected deer herds. Infection of cattle by wild deer also apparently occurred in two instances, one at Timahanga and the other at Waikaka, both referred to earlier.

Conclusion

Suppurating sinuses, a common feature of the disease in deer, are probably responsible for a large proportion of deer to deer transmission. The preponderance of lesions in the medial retropharyngeal lymph nodes suggests that most bacillary uptake occurs from the oral cavity, probably via the tonsillar lymphoepithelium. Experimental inoculation of the tonsillar crypts has been shown to reliably reproduce a disease state similar to that seen naturally, thus substantiating the primary importance of the oral route of infection. Coughing is not commonly observed in healthy deer, nor in those with pulmonary tuberculosis, and thus aerosol transmission is likely to be a secondary transmission mechanism only. This is in contrast to cattle where thoracic lesions predominate, and coughing is a marked feature of the disease.

The majority of infected deer in New Zealand have single lesions containing few AFB, suggesting that most have a degree of resistance to the disease, and that they probably shed few bacilli. The transmission of disease between deer is typically slow unless the animals are subjected to stressors and heightened adrenal

glucocorticoid responses. In susceptible animals this allows disease progression, and bacillary excretion to escalate, especially from draining abscesses.

There is compelling evidence from New Zealand and overseas studies to substantiate that deer to cattle transmission, does occur, albeit infrequently. However, the exact mechanism is still unclear, but likely to involve close direct contact, as the probability of environmental survival of bacilli in sufficient numbers to establish disease is likely to be low (Jackson *et al.*, 1995).

New Zealand, British and North American data suggests that the disease exists at very low prevalence in free-ranging deer populations for prolonged periods in the absence of other sources of infection. In Britain and Ireland, where infected badgers are found, the prevalence of tuberculosis in deer is higher, but still probably between 1 and 5%, which suggests that there is some transmission from badgers to deer, as well as to cattle. In New Zealand the 20 to 30% prevalence in wild deer in areas with endemic tuberculosis suggests the involvement of tuberculous possums in maintaining the high prevalence of disease. This suggestion is supported by the observed interactions of sedated possums and deer, and by the slow spread of tuberculosis in the Turangi area, where the disease seems to have remained confined to the area with tuberculous possums, despite the potential existing for it to have spread widely with deer dispersal.

Possoms (*Trichosurus vulpecula*)

This review of the tuberculosis in possums, a reservoir host (Morris and Pfeiffer, 1995), is not intended to be comprehensive, but is focused on some aspects of the disease which are relevant to other parts of this thesis. For a more complete review of possum ecology and involvement with tuberculosis the reader is referred to Jackson (1995).

Prevalence

Prevalence of disease varies considerably between populations and at different times in the one population. Reported tuberculosis prevalence from cross-sectional studies often ranges from less than 1% to 10% (Cook, undated; Coleman, 1988; Nugent, 1994; Fraser *et al.*, 1995; Pfeiffer *et al.*, 1995; Caley, 1995b). At Flagstaff Flat on the West Coast the prevalence of gross lesions was found to be 6.98% in 1980, whereas in 1992, 50.0% of possums had tuberculous lesions despite the

density of possums being apparently five times lower than in 1980. At this site the prevalence has fallen steadily in subsequent years, such that 4 years after the initial high figure, the prevalence was found to be less than 1.0 % in 1996, and the density of possums over the same period has been steadily increasing (J. Coleman, pers. comm.). In many infected populations with a low prevalence of disease, no grossly affected animals are found at necropsy despite hundreds or thousands of animals being examined. This suggests that the disease can persist when the prevalence of infected individuals is extremely low, or that a proportion of possums are non-lesioned carriers of the disease, and that the disease can persist in these carriers for a prolonged period. Bell (1974) found that the disease can persist when possums are at low population densities of around 2 per ha.

Pfeiffer (1995) found no correlation between possum density indices and prevalence of tuberculosis in the Hauhungaroa Ranges. However, Coleman (1988) found the highest prevalence of tuberculous possums (33.3%) in the Hohonu Range was in areas with rough pastoral grazing, where there was a low density of possums, which were able to be readily trapped to extinction. Later observations made by Coleman *et al.*, (1994a) also found the highest prevalence of tuberculosis in possums associated with sub-maximal densities at Flagstaff Flat on the West Coast. The highest prevalence of disease (20%) at the Castlepoint study site was also found to be associated with the lowest population density (Jackson, 1995). The apparent absence of human influence on the populations cited, suggests that either tuberculosis or some other adverse environmental factor, or both, was responsible for the sub-maximal possum densities in those areas.

Clustering

Infected possums are often spatially localised and separated by populations apparently free of tuberculosis (Coleman, 1988). Clustering of the disease in 'hot spots' has been observed in numerous studies and trapping operations, although this is not invariably the case, as Coleman *et al.*, (1994a) found tuberculous possums occurring more or less continuously along several kilometres of river frontage at Flagstaff Flat. These clusters of diseased individuals are believed to occur on a "micro" geographic scale, with diameters measured in tens of metres (G. Hickling pers. comm.), with Pfeiffer *et al.*, (1995) calculating an average cluster size of 32.6 m, and an average prevalence of infection of 76% in those clusters. There is some

evidence that these clusters of infection persist over time (Hickling, 1991; Morris and Pfeiffer, 1995) and are associated with local aggregations of animals, where possums are 4-16 times as crowded as if they were distributed randomly in the environment (Hickling, 1995).

It has been suggested by Morris and Pfeiffer (1995) that this disease aggregation arises as a consequence of one of two processes; 1) either, the continued presence of infected possums, which must remain infectious for lengthy periods, or persistence of infection in latent carriers for a prolonged period; or 2) environmental persistence of *M. bovis* in sites both favourable to the organism and for indirect transmission e.g. den sites. If persistence of the organism in den sites were responsible for the maintenance of tuberculosis, then theoretically the clusters of disease should continue to expand around the favourable environment, but this has not been observed in the field. Studies of environmental persistence (Jackson *et al.*, 1995c) have also shown that even in dens, bacilli are unlikely to remain viable for over 3 weeks. Persistence of infection in possums may be responsible for the development of clusters where pseudo-vertical disease transmission, aggregated mating patterns, or environmentally stressful areas are found (Pfeiffer, 1994; Morris and Pfeiffer, 1995), or possibly, where habitat factors favour disease transmission at particular sites e.g. heavily used dens, or where there are food sources at which possums congregate to feed (Hickling, 1991). Of these hypothesised causes of hot spots, only pseudo-vertical transmission has been confirmed to occur (Jackson *et al.*, 1995b). In some areas e.g. Hawkes Bay several possums are known to simultaneously share the same den site, such as a hollow log (Fairweather *et al.*, 1987). This close and intimate association is almost certain to favour transmission of tuberculosis in environments where suitable sites, such as hollow logs or cavities beneath tree roots, can be found, and there is a shortage of other suitable shelter. In other areas, e.g. around Castlepoint, simultaneous den sharing is uncommon (Paterson *et al.*, 1995), and even the use of another possum's den site on days when the normal resident is absent also seems to be rare (C. Sauter, pers. comm.). Thus in some environments den sharing is unlikely to be responsible for substantial transmission of tuberculosis between possums. The most plausible explanation for the persistence of infection in clusters of possums is the continued presence of *M. bovis* possums.

In an attempt to establish whether unfavourable environmental variables affected the establishment of spatial clusters, Caley (1996) examined and compared the characteristics of possum den sites from areas where tuberculous possums had, or had not been captured over a number of years, in a farmland area near Taumarunui. The results failed to demonstrate any significant difference in the measured quality of den sites used by possums. The conclusion of the study was that if den quality does play a role in the origin of tuberculous clusters of possums, then that quality difference is difficult to detect, especially with tests of low-to-moderate power which were used by Caley. The occurrence of possum crowding at 'hot spots' suggests that environmental conditions favourable to possums exist in these areas, rather than the reverse, as suggested by Morris and Pfeiffer (1995).

Sex and age effects

Coleman (1989) found no overall significant difference in the prevalence of infection between males and females in the Hohonu Range, although significantly more immature males than females were infected. Pfeiffer (1995) found a similar pattern in the Hauhungaroa Range, where juvenile males had a higher prevalence of disease than juvenile females, but with the difference only approaching statistical significance. In a series of cross-sectionally sampled possums, Jackson *et al.*, (1995a) found that of the variables sex, age, amount of fat reserves and weight, only sex was a significant risk factor for the occurrence of tuberculosis infection, with the relative risk for males being 1.78 times that of females. This male bias has not been found in the Castlepoint study, where Jackson *et al.*, (1995a) reported a similar prevalence of disease detected in both males and females. This prompted Jackson *et al.* to suggest that a trapping bias, which reduced the number of infected females captured, may have contributed to the higher prevalence observed in males in previous cross-sectional studies. However, the diagnostic methods used by Jackson *et al.*, (1995a), which were based upon clinical examination, may also have had inherent, but unknown biases, which may have reduced any differences in sex-related prevalence. No correlation between the sex or presence of pouch young, and the number of tuberculous lesions in possums was found Jackson *et al.*, (1995a). Taken together, this data suggests that the disease process operates similarly in either sex, but that variation in prevalence observed between males and females involves behavioural rather than intrinsic physiological factors. This further suggests that in some environments, where males have been found to have a higher

prevalence of tuberculosis than females, that they are exposed to a greater number of risk factors for disease development, which might include aggressive encounters, territory marking and the maintenance of larger activity areas (Jackson *et al.*, 1995b; Paterson *et al.*, 1995). For immature males this increased risk may relate to the stresses and interactions (possibly infectious) involved with dispersal movements, which commonly occur between 9 months and 2 years of age (Cowan and Rhodes, 1993; Efford, 1991). Males are far more likely to disperse, and may suffer a reduced chance of survival as a consequence (Ward, 1985; Brockie, 1991; Paterson *et al.*, 1995).

Seasonal effects

Coleman (1988) found significant differences in prevalence of disease between bimonthly sampling times in the Hohonu Ranges. A higher prevalence of infection existed during the autumn and winter in animals captured on the pasture edge, but not in possums captured in the forest. In the Hauhungaroa Ranges, Pfeiffer *et al.*, (1995) found the prevalence in immature possums increased from 1.5% in the early summer to 3.8% in mid summer, to 4.17% in late summer, whereas a similar trend was not seen in mature possums. At Castlepoint, Jackson (1995) found the prevalence of tuberculosis in possums was lowest from February to June, and was highest in November.

From the few reports noted above, it appears that seasonal influences on disease prevalence do exist, but that seasonal peaks vary between different environments and age groups of possums. This suggests that the processes which drive the development of disease are subject to considerable variability, both temporal and spatial.

Pathology

Generalised, rapidly progressive and fatal disease has been shown to follow experimental inoculation of possums with *M. bovis* (Bolliger and Bolliger, 1948; Corner and Presidente, 1980 and 1981; O'Hara *et al.*, 1976; Buddle *et al.*, 1994). In these studies mortality occurred between 25 and 100 days post-inoculation. The rapid progression of disease in experimentally infected possums may be due to the combined effects of high infective dose, route of administration, and stress of captivity (Jackson *et al.*, 1995a), and hence may not accurately reflect the speed of disease development in naturally acquired infections of free-ranging possums. In

the naturally infected Castlepoint study population most possums identified as clinically tuberculous died within 2 months, but a small number have survived from between 6 and 22 months (Jackson *et al.*, 1995a). This suggests that at least some possums which become diseased in the wild suffer a prolonged course of infection. The scarcity of information regarding the time of infection and development of the disease in possums under natural conditions allows very limited speculation regarding the potential for disease establishment following contact with *M. bovis*, rates of lesion development, or the likelihood of lesion resolution/regression.

Apart from the lung and associated draining lymph nodes, the axillary and inguinal lymphocentres have been found to be the most frequently diseased sites in tuberculous possums (Jackson *et al.*, 1995). However, the means by which the axillary and inguinal lymphocentres become infected is not well understood. It has been suggested that there may be an obscure, and as yet unidentified lymphatic link between the lung and the left axillary lymphocentre which has an inexplicably high prevalence of infection (Jackson *et al.*, 1995b.). Alternative explanations are that the axillary lymph nodes are simply predilection sites for lodgement and multiplication of the organism, or that inapparent primary skin infection occurs in association with fight wounds or sternal gland rubbing on scent-marked trees (Jackson *et al.*, 1995b). Although subcutaneous tuberculous abscesses, independent of known lymphatic tissue sites, are occasionally seen (unpublished data), they are uncommon, but could lead to lesion development in the axillary or inguinal lymphocentres and may be caused by percutaneous infection. However, Jackson *et al.*, (1995b), points out that evidence for primary skin infection causing lesions in the inguinal or axillary lymphocentres is not strong, as it is likely to be principally associated with sternal gland rubbing in mature males only, but there has been no association found between sex or age, and infection at these sites. Moreover, superficial lymph node sites were involved in five of six juvenile possums with early stage disease, prior to sexual maturity, when fighting and territorial marking behaviour normally begins.

Possums with lesions in lung, inguinal axillary lymph nodes were found by Jackson *et al.*, (1995a) to be 1.4 - 1.6 times as likely to have evidence of haematogenous spread as animals which did not. This suggests that infection at these sites are either the source, or the result of, haematogenous dissemination. As lesions were

found in sites which could only have been infected by blood-borne bacilli, in 86% of tuberculous cases, Jackson *et al.*, (1995a) concluded that haematogenous dissemination occurs early in the course of the disease in possums, and is the most likely route of infection for the axillary and inguinal lymphocentres (Jackson *et al.*, 1995b).

Routes of transmission

In experimental studies, natural infection has been observed in un-inoculated possums caged separately from, but housed in the same room as other tuberculous possums (O'Hara *et al.*, 1976; Corner and Presidente, 1981; Buddle *et al.*, 1994), and in one instance a cat (Isaac *et al.*, 1983). Transmission was believed to have occurred by infectious aerosols (Jackson *et al.*, 1995a), but other infected airborne particles may have been involved, as in each situation animals with draining tuberculous sinuses were present.

The high prevalence of disease in the axillary, inguinal lymphocentres and the lungs suggests an important role for these sites in disease transmission. Jackson *et al.*, (1995b) isolated *M. bovis* from 36% of the tracheal washings of tuberculous possums. P. Livingstone (pers. comm.) also isolated bacilli from 19 of 51 pharyngeal swabs taken from tuberculous possums, from six of 51 faecal samples and from one of 14 urine samples. Jackson also isolated *M. bovis* from the faeces and urine of one of three terminal cases. Despite tuberculous lesions being found in 45% of kidneys, excretion via the urine appears to be uncommon, whereas the isolation of *M. bovis* from tracheal or pharyngeal samples is common, and suggests that respiratory/oral secretion will be important in disease transmission.

Tuberculous cutaneous fistulae were found in 31% of infected possums by Jackson *et al.*, (1995b), these often drained lesions in the axillary and inguinal lymphocentre. Possums with discharging sinuses had significantly more lesion sites than infected animals without sinuses, which prompted the conclusion that suppurating sinuses, as a whole, were confined to animals with advanced disease. Fastidious grooming by possums is likely to contaminate the oral cavity of those with suppurating sinuses, if not already contaminated by bacilli brought up from the lungs or excreted by infected tonsils (Jackson *et al.*, 1995b). Bacilli discharged from these fistulae will contaminate the environment, and other possums in which

there is physical contact, thus potentially providing a common means of both direct, and indirect, transmission to other possums.

Four females were found by Jackson *et al.*, (1995b) to have tuberculous lesions in the mammary gland, and *M. bovis* was recovered from tissues of a 70-day-old pouch young. Two other dependent young were also examined histologically and found to have tuberculous lesions in the lung and lymphatic tissues. These findings strongly support the hypothesised occurrence of pseudo-vertical transmission to pouch young (Morris and Pfeiffer, 1995; Jackson, 1995). It is believed that the risk of pouch young infection is high, as no other segment of the population is exposed to infection for as long or from as many potential routes of transmission.

As definitive primary complexes or single site lesions were not found in the possums examined by Jackson *et al.*, (1995b), they were unable to pin-point the portals of bacillary entry. This suggests that the initial focus of infection is poorly contained, and that a rapid generalisation of infection occurs, with subsequent lesion development in predilection sites. It is clear that organisms are principally available for excretion from the lung, suppurating sinuses and possibly the oral cavity. However, the actual demonstration, or quantification of excretion by these routes, is presently lacking. The data does suggest that the respiratory tract is the major excretion route in adults, and by inference from other species, also the principal site for initiation of infection. Juvenile animals are most likely to be infected by pseudo-vertical transmission, whereas mature animals are more likely to acquire infection directly from agonistic encounters, mating or concurrent den sharing activity, and less frequently by indirect means such as territorial marking, or self-grooming following the use of a contaminated den.

Pigs (Sus scrofa)

Ecology

Feral pigs are widespread on both the North and South Islands, living in areas with suitable water and food supplies, and adequate cover (McIlroy, 1990). They are currently generally considered to be a minor, often localised pest, with numbers usually controlled by recreational hunting.

A definite breeding season is absent, but there is a peak in births in the spring and summer, with litter size ranging from one to ten piglets (McIlroy, 1989), with it being unusual for more than six offspring to survive. The piglets stay near the nest site for the first 2 to 3 weeks following birth, and thereafter keep in close contact with the sow by frequent nose to nose touching (McIlroy, 1990).

In the Murchison area, pigs reached local densities of up to 43 per km² in good undisturbed habitat of broken forest, and home ranges varied between 28 and 209 ha, with the larger ranges being covered by juveniles. In the Central Otago area, with open habitat, home ranges for sows were estimated to vary between 543 and 2341 ha, and for boars to vary between 2257 and 15717 ha (Knowles, 1994). In this habitat both the boars and sows were known to move up to 8 km between tracking episodes, with the exception of one boar which moved over an area 20 km length. In better habitat, with adequate cover sows are likely to move distances of only 0.5 km, and boars up to 3.2 km within their normal ranges (McIlroy, 1990). Pigs tend to be gregarious and gather in small social groups, except for boars over 18 months old, which lead a more solitary existence.

Feral pigs are omnivorous, opportunistic feeders, living mainly on a high fibre/low protein diet. They readily eat other animals when available. In a dietary study in the podocarp-tawa forest of the Urawera Ranges (Thomson and Challies, 1988), possum carrion was found to form 10.6% (by dry weight) of feral pig diet. Possum consumption occurred in all seasons, but the majority was eaten in winter and spring. Pig carrion comprised 1.8% of the diet, and there was also a small contribution from red deer.

Disease in pigs

It is believed that, of all the domestic species, the pig is the most susceptible to infection with *M. bovis* (Francis, 1958). Prior to the implementation of bovine tuberculosis eradication programmes in cattle *M. bovis* infection in pigs was very common, and thought to have been related not only to the ingestion of milk from tuberculous cattle (Francis, 1958), but also to rooting and feeding around contaminated cattle dung (Albiston *et al.*, 1954). Although disease (detected at slaughter) used to be prevalent in domestic pigs, with some countries reporting over 30% of slaughter stock with tuberculous lesions (both avian and bovine types), clinical cases were rare (Albiston and Pullar 1954). The disease is particularly

severe in young pigs, with lesions in the tonsils, head lymph nodes, lungs and abdominal organs two to three times more severe than those found in calves given the same dose orally (Griffith, 1907). However, Griffith (1907) also found that doses of 0.1mg of *M. bovis*, given subcutaneously to 19 - 21 week old pigs were not regularly lethal. By comparison with juvenile animals, mature pigs are resistant to the disease. This was demonstrated by Luke (1951), who showed that some mature pigs were able to resist even large doses of *M. bovis* administered intraperitoneally.

Lymph node lesions initially show nodal enlargement with caseation and softening, and later become calcified. Peripheral reaction is marked, and composed of varying amounts of fibrous tissue and lymphocytic infiltration. With time the lesions often reduce in size to such an extent that the node will appear normal, whilst still containing small gritty caseous nodules surrounded by thin fibrous capsules (Cornell and Griffith, 1930). Older sows and boars tend to show disease typified by fibrosis and calcification, the lesions containing few AFB (Francis, 1958). Observations suggest that the overall effect is for lesions to regress as the pig ages and time from infection increases (Ray *et al.*, 1972). *M. bovis* has been recovered from five of 33 lymph nodes without gross lesions, which had been removed from 22 pigs with severe or generalised disease (Feldman, 1936), thus demonstrating that “a normal appearing lymph node is no assurance of the absence of tubercle bacilli”. Ray *et al.*, (1972) also found that it was common to isolate *M. bovis* from non-lesioned lymph nodes, and attributed this to the early generalisation of infection, with subsequent resolution of the disease. In New Zealand, a small but significant proportion of tubercle-like lesions in domestic pigs fail to yield mycobacteria (Nuttall, 1986). It was suggested that mycobacteria may have been present in extremely low numbers in lesions, especially those of older animals, and that other bacteria, particularly *Rhodococcus equi*, but also actinobacilli, staphylococci, or streptococci may have caused a small proportion of the lesions.

In summarising the data from several studies on domestic pigs, Francis (1958) showed that lesions are most common in the head lymph nodes (68%), mesenteric lymph nodes (65%), respiratory lymph nodes (58%), lungs (51%), liver (38%), and spleen (22%), of diseased animals. Tonsils were infected in 13% of pigs, and lesions were also occasionally found in other body nodes, kidneys, joints and bones.

In farmed pigs in New Zealand, Nuttall (1986) found the most commonly involved sites to be the mandibular, retropharyngeal, cervical, and inguinal lymph nodes.

Lesions in the mammary glands and uterus have been reported, with mammary disease occurring in 5% of generalised cases (Francis, 1958). Despite the mesenteric lymph nodes being very commonly affected, tuberculous intestinal ulcers are rare.

M'Fadyean (1915, cited by Francis, 1958), also reported that 3.75% of unweaned pigs were infected with tuberculosis, suggesting that they were infected by their mothers. Albiston *et al.* (1954) also reported 8 of 17 unweaned piglets from two litters, infected with *M. bovis* in Australia. Suppurating tuberculous sinuses have been reported by Plum and Slynborg (1938, cited by Francis, 1958), and these investigators also successfully isolated *M. bovis* from the tracheal mucus of 24% of 96 pigs with extensive tuberculous lesions. Ray *et al.*, (1972) also demonstrated transmission of *M. bovis* from three pigs with widespread disease following an oral inoculation of 2 mg of *M. bovis*, to three non-infected pen mates, in a trial investigating the pathology of mycobacterial disease in pigs.

Despite the fact that disease generalises rapidly in pigs, and there is no reason to doubt that the route of infection in nearly all cases is by the alimentary tract (Francis, 1958). Chaussé (1915, cited by Francis 1958) believed that 80% of pigs are infected via the tonsils, despite lesions at the site of entry being difficult to detect. Early lesions took the form of small mucosal elevations centred around crypt entrances, followed later by caseation, ulceration in some cases, and eventually resolution and scarring. The lymphatic drainage of the soft palatine tonsil of pigs has recently been described in detail by Belz and Heath (1995b). There were two drainage pathways identified; a superficial route which drained laterally to the mandibular and accessory mandibular nodes, and thence to the ventral and dorsal superficial cervical lymph nodes, and a deeper route which drained more directly to the medial retropharyngeal node and thence to the tracheal trunk. The widespread involvement of multiple head lymph nodes with tuberculosis infection is thus readily explained by primary tonsillar uptake and subsequent distribution in efferent lymphatics. Primary respiratory infection is rare in pigs but has been recorded, and it is believed that the vast majority of pulmonary lesions occur subsequent to haematogenous dissemination.

Tuberculosis in wild pigs internationally

There is no documented case of tuberculosis persisting in wild pigs or wild boar in the absence of infection in other species. On the Hawaiian island of Molokai, *M. bovis* was isolated from nine (15%) of 61 wild pigs, and on combining results of histopathological examination and culture, the prevalence was estimated to be approximately 20%. Tuberculosis in these pigs was associated with the presence of infection in cattle and axis deer (*Axis axis*) in the same area (Essey *et al.*, 1981). In 1992 it was reported that tuberculosis due to *M. bovis* was confirmed in 9 (20%) of 44 wild boar shot in Hungary through the period from 1985 to 1991 (Kormendy, 1993, cited by O'Reilly and Daborn, 1995). Tuberculosis is prevalent in both cattle and deer herds in Hungary. In California it was speculated that feral pigs were responsible for the maintenance of infection in a ranch holding 4000 head of cattle (Allison, 1967). The disease was thought to have been transmitted through contaminated pig wallows at which cattle congregated in summer. Successful eradication of tuberculosis followed the slaughter of over 90% of the pigs, and a test and slaughter programme of the cattle, spanning several years (Knowles, 1994).

The history of *M. bovis* infection in wild pigs in Australia has been well documented only in the floodplain habitats of the Northern Territory, where pig numbers are high, and interaction with feral cattle and water buffalo (*Bubalis bubalis*) is common. Between 1958 and 1964, 260 pigs from various places in the Northern Territory were examined, with 85.4% found to have gross lesions resembling tuberculosis. *Mycobacterium bovis* was cultured from 54.4% of 149 lesions (Letts, 1964). The prevalence of lesions ascribed to *M. bovis* infection was 46.5%. Corner *et al.*, (1981) found gross lesions characteristic of *M. bovis* infection in 47.7% of 751 pigs sampled between 1973 and 1976. Histopathological examination showed that mycobacterial granulomas comprised 57% (102 of 179) of the macroscopic lesions investigated. Of 202 pigs examined bacteriologically, 37 (18.3%) provided isolates of *M. bovis*. It was suggested that the overall prevalence of *M. bovis* infection was 19%. There was a 8.2% prevalence of generalised disease in the 61 tuberculous pigs which were subjected to complete necropsy. In 38 pigs the only lesion was in a mandibular lymph node. An inguinal lymph node from two animals, and two uteri and one testicle showed gross lesions, but no pigs with pulmonary lesions were detected. It was suggested that because the prevalence of lesions increased with the age of the pig, yet the success of culture reduced with

age, that the pigs were continually exposed to infection, but with age became increasingly capable of overcoming the infection and destroying bacilli in lesions.

The moderate prevalence of tuberculosis in the pigs of the Northern Territory was believed to have arisen through close association with wild cattle and water buffalo, which have similar environmental requirements. The pigs in particular, are known to feed on the carcasses of cattle and buffalo, which commonly die from starvation and bogging at the end of the dry season. The mean prevalence of tuberculosis in buffalo at abattoir inspection was 16.4% (range 8.0% - 25.5%) for 11,909 buffalo bulls slaughtered between 1959 and 1964 (Letts, 1964). The prevalence reduced over time, so that in a later study prevalence was determined to be 8.0% (range 6.3% - 15.6%) for 33,755 animals slaughtered at four abattoirs between 1966 and 1974 (McCool and Newton-Tabrett, 1979) and only 1.7% (range 0.3% - 8.2%) of 11,322 animals from 17 farms slaughtered in 1979 (Hein and Tomasovic, 1981). Since 1985 the numbers of feral water buffalo and wild cattle have been considerably reduced due to the activities of the National Brucellosis and Tuberculosis Eradication Campaign.

McInerney *et al.* (1995) undertook further studies of feral pigs in the Northern Territory in 1992, to establish whether the reduction in the prevalence of infected buffalo and cattle had brought about an associated reduction in the prevalence of tuberculosis in wild pigs. Pigs were taken from five of the six sites surveyed by Corner *et al.* (1981). Destocking of the areas in which the pigs were sampled had reduced the density of potentially infected cattle and buffalo from the 1973-76 level of 10 - 50 beasts per km² to between 0.0 and 0.1 per km². Of the 790 pigs examined in 1992, 49 had lesions grossly resembling tuberculosis. All gross lesions were subjected to histopathological examination and mycobacterial culture. One boar was found to have a mycobacterial granuloma, and *M. bovis* was recovered from another sow and boar. The observed prevalence of both confirmed tuberculosis (0.25%) and gross tuberculous lesions (6.2%) was significantly less than the estimated prevalence of infected (19%) and gross tuberculosis lesions (47.7%) found by Corner *et al.* (1981) 16 years earlier.

The lack of generalised disease, and the moderate prevalence observed in the feral pigs of the Northern Territory in the earlier cross-sectional studies may have arisen through only the more disease resistant older juveniles (4 to 5 months of age) and

mature pigs, principally having access to tuberculous carcasses at the end of the dry season. Despite the moderate prevalence of infection present in buffalo, many of the carcasses may have had low bacillary loads as lesions in cattle and buffalo usually contain few AFB (L. Corner pers. comm.). The high temperatures generated during putrefaction in these large dark skinned animals in a tropical environment may further reduce the number of mycobacteria present before scavenger ingestion.

Tuberculosis in wild pigs of New Zealand

In 1970 Ekdahl *et al.* reported 14 cases of tuberculosis in feral pigs dating back to 1964. *Mycobacterium bovis* was isolated from all 13 of the pigs cultured. These early cases came from near Wellington, and also from North Canterbury and Westland. Since then *M. bovis* infected pigs have been found in all of the major endemic areas, including the Woodhill State Forest, Wairarapa, Central North Island, Otago and Southland (de Lisle, 1994). From 1987 to 1993 the Tuberculosis Laboratory at Wallaceville isolated *M. bovis* from 105 of 246 (42.7%) samples from feral pigs (de Lisle, 1994).

Farmed pigs from within the endemic areas are commonly infected with tuberculosis, usually with lesions in the head and gut-associated lymph nodes, suggesting that infection was by the alimentary tract (Nuttall, 1986). Feeding of infected possums was suggested as the cause of a tuberculosis outbreak in twenty five, 6-8 month old domestic pigs (McLaughlin, 1989). Lesions in diseased pigs were principally identified in the mandibular lymph nodes, but generalisation was common, and resulted in the condemnation of nine of the pigs at slaughter. *Mycobacterium bovis* has been isolated from pigs taken from most endemic areas, and also from pigs sampled from outside recognised endemic areas, e.g. a tuberculous pig found in Northland in 1991 (de Lisle, 1994). It was suggested that the transport and live release of feral pigs by hunters may have been responsible for this unusual case.

Wakelin and Churchman (1991) reported a prevalence of gross tuberculous lesions in feral pigs of 31% in 251 pigs examined from the Pisa Range area of Central Otago. Of 41 (16%) with histologically positive lesions, 16 (39%) were found to harbour *M. bovis*, thus providing a prevalence of bacteriologically confirmed infection of 12.0%. Of the pigs with histologically confirmed tuberculosis, 96%

had involvement of one or more head lymph nodes. In addition, 33% had lung or respiratory lymph node involvement, and 21 animals were found to have generalised disease, with lesions in four or more sites. The prevalence of disease increased with age, being 24% in pigs younger than 2 years old, and 42% in the remainder. In 1990 the annual incidence of rates of infection in farmed cattle and deer in this area were 1.18% and 0.34% respectively.

A further cross-sectional study was conducted in the Central Otago area between 1991 and 1993 (Knowles, 1994). In this exercise radio-collared judas pigs were used to track and shoot 85 pigs in the Pisa special tuberculosis investigation area. Histological evidence of disease was present in 31.8% of the pigs, with the vast majority of lesions present in the mandibular lymph node. *Mycobacterium bovis* was isolated from 17 (20.0%) of the 85 animals necropsied. Outside of the special tuberculosis investigation area, none of 59 pigs shot showed evidence of tuberculosis, although *M. bovis* was isolated from lesions found in two animals shot independently by hunters in this area during the same period. No cases of generalised tuberculosis were identified, and tuberculosis appeared to be clustered in family groups, with some or all piglets, even down to body weights of 5 kg (approximate age 4-6 weeks), shot with infected sows having tuberculous lesions. These observations suggest a possibility of pseudo-vertical transmission in at least some family groups. An alternative explanation may have been group contact with a single point source of infection. It is believed that tuberculous deer escaped from captivity in the Pisa Ranges through the period between 1982 and 1984. However tuberculous wildlife were not implicated in herd breakdowns until 1989 and later. The low prevalence of tuberculosis in livestock and the occurrence of a small number of infected ferrets and possums in the area suggests that most of the infection in the pigs has arisen from consumption of infected carrion. However the possibility of some intraspecific transmission cannot be discounted, owing to the occurrence of disease in very young pigs.

Conclusions

Most lesions seen in pigs seem to be the result of infection via the alimentary tract, as a result of the consumption of tuberculous material. The oropharyngeal tonsil is likely to be the principal site of mycobacterial entry to the body, and from there will spread to the draining lymph nodes of the head, and further afield.

Although pigs are readily infected, they are unlikely to die from the disease unless very young, and the infecting dose high. With time some lesions may resolve, so that such sites will become inconspicuous, and contain few bacilli, thus making isolation of the organism difficult. Isolation of bacilli from apparently non-lesioned sites is likely to be a common occurrence.

Pseudo-vertical transmission is possible, from mammary lesions, respiratory excretion and draining abscesses. Despite pigs being gregarious creatures, and horizontal transmission being possible through a variety of pathways, i.e. respiratory excretion, draining abscesses, sexual contact and urine, the overall contribution of these mechanisms to the acquisition of disease amongst pigs seems only minor compared to the ingestion of tuberculous material.

In the Northern Territory the prevalence in pigs appears to be linked to the amount of tuberculous cattle and buffalo carrion available. The disease in this area was characterised by lack of generalisation, and failure to isolate the organism from many of the lesions suspected as having a tuberculous aetiology. The eradication of tuberculosis in Australia as a whole, was not apparently hindered by the presence of tuberculous pigs in areas where tuberculosis in cattle and feral pigs were both common. Despite the suspicion that feral pigs may have been involved in the transmission of tuberculosis to cattle in a Californian herd, there was no substantiation of these claims. In this herd of 4000 head of cattle there were likely to have been dead tuberculous cattle available for consumption by the pigs on many occasions, and tuberculosis eradication hindered by the size of the herd, rather than by transmission of disease from tuberculous pigs. Although transmission from pigs to cattle has been suspected on a number of occasions, there have always been other more plausible reasons for breakdowns occurring in the herds, and adequate evidence of pig to cattle transmission has never been shown. The pig removal trial in Central Otago, which investigated the effect on livestock reactor rates, yielded inconclusive results (Caley, 1994) and pen studies in the Northern Territory investigating transmission of tuberculosis from infected pigs to cattle failed to provide evidence of transmission (P. Caley, pers. comm.).

In New Zealand possum carcasses are commonly eaten by pigs, and of the tuberculous wildlife available, probably provide the most common source of infection. Unfortunately the only cross-sectional studies so far reported have both

come from the same area in Central Otago, an area in which tuberculous carrion is not common, and one where the behaviour of pigs may not be representative of many other areas in New Zealand. There is some evidence supporting a role for pseudo-vertical transmission, there is potential for transmission via cannibalism, but the likelihood of pigs with generalised disease excreting sufficient bacilli to infect cohorts seems slight given the data from existing studies. There is a dearth of knowledge on the disease in feral pigs in this country, and it may be unwise to conclude that the epidemiology of the disease will prove to be the same as in Australia or other countries where control has disregarded the tuberculosis status of pigs, and the disease has been successfully eradicated. It is not known whether pigs could be a reservoir species for *M. bovis* infection in New Zealand, but the possibility seems remote. Due to their scavenging habits, and the ease with which they become infected with *M. bovis*, and display gross lesions, feral pigs may be regarded as useful indicator species of *M. bovis* infection of other wild animals in an area. A Game Packing House at Rotorua has recently commenced processing feral pigs (Weston, pers. comm.), and could provide an ideal opportunity to monitor the distribution and prevalence of tuberculosis in pigs from the central North Island area.

Sheep (Ovis aries)

Tuberculosis has traditionally been considered rare in sheep and most published information regarding the disease concerns isolated cases only. Historically the reported prevalence in sheep has always been very low, and well under 1% (Francis, 1958). Lesions are characterised by a similar appearance to those of cattle, and are often calcified. The distribution of lesions is also similar to cattle, with a predominance of lung and thoracic lymph node involvement. Lesions in the udder have been found in one sheep by Murphy (1935). The gross appearance is also similar to caseous lymphadenitis (pseudotuberculosis or CLA), and may lead to mistaken diagnoses if identification is based on gross lesion appearance only. Carmichael (1938a) noted that amongst a group of Ugandan sheep, a striking feature of the disease was a lack of clinical signs, despite the presence of severe pulmonary lesions. This may be somewhat analogous to deer which also fail to show coughing as a feature of advanced pulmonary infection, and which as a consequence appear to have limited intraspecific transmission by the respiratory route. Although there is little information available on how sheep acquire infection,

one report from the USA (Essey, 1991) suggested that tuberculosis found in a group of exotic breed sheep was contracted through contact with a deer herd with a high prevalence of infection.

Although only a limited number of observations on the susceptibility of sheep to experimental inoculation with *M. bovis* have been performed, it seems that their susceptibility is similar to cattle (Koch and Schutz, 1902, cited by Luke, 1958; Francis, 1958; Wilson and Miles, 1975). This has recently been confirmed by the findings of a severe outbreak of tuberculosis caused by *M. bovis* in a sheep flock in Germany (Uhl and Müller, 1995). Of 480 animals subjected to intrapalpebral tuberculin injection, 55% showed positive reactions. Some of the tuberculous animals showed signs of wasting, and of 15 animals necropsied, 12 showed severe lesions. No cause of the outbreak could be determined, although it was believed that it may have been contracted from a common point source which infected all sheep simultaneously. The epidemic was apparently unrelated to infection of cattle, neighbouring sheep flocks and sheep dogs.

Occasional ovine cases of tuberculosis have been reported in New Zealand. Black and Orr (1996) reported a sheep with caseous lesions of the mesenteric and hepatic lymph nodes and liver, found at routine meat inspection, from which *M. bovis* was isolated and a few AFB were seen on sections of typical tuberculous lesions. J. Adams (pers. comm.) has also found several cases of tuberculosis in older sheep grazing within the central North Island endemic area, and G. Pannett (pers. comm.) has also identified the occasional tuberculous sheep in the Wairarapa endemic area. However, these few reports may not represent the true situation in this country as there have been two earlier reports which suggest that in a few cases mature sheep from flocks grazed within endemic areas may have a moderate prevalence of disease, although the risk factors for this were not indentified.

Davidson *et al.*, (1981) tuberculin tested a mob of 596 mixed age ewes, which had been grazing with infected cattle in the West Coast endemic area. Eighteen percent of the mob reacted to intradermal tuberculin injection, and of these reactors, 70 were examined by necropsy. Tuberculous lesions were found in 43 (61.4%) of the sheep, with lesions commonly found in the abdominal cavity and caudo-dorsal lung lobes and thoracic lymph nodes. The lesions varied from areas of yellow-grey caseous necrosis through to heavily calcified caseous encapsulated lesions, which were

similar to those seen in cattle. Acid-fast bacilli, although difficult to find were present in ten of the 43 cases. From the distribution of lesions it was determined that about half of the infections were caused by ingestion of bacilli, and the other half by inhalation. Although concern was expressed over the potential for respiratory excretion, as eight of the 70 necropsied had lesions in the lung parenchyma, there was no discussion of how the sheep may have acquired infection.

From another farm in the central North Island endemic area, 286 mixed-age sheep were examined for evidence of *M. bovis* infection (Cordes *et al.*, 1981). Forty three (15.0%) of the sheep possessed gross lesions resembling tuberculosis, and from these, tubercle bacilli were demonstrated histologically or by culture in 32 (11.2%). The lesions were typical of those already described for sheep, but 26 which were subjected to detailed necropsy, were remarkable in this instance for the great number showing generalised lesions. In 20 of the 26 the liver or spleen were involved, in 23 the thoracic tissues were involved and in 21 there were intestine-associated lesions. In 3 of 10 sheep with bronchial swabs cultured, isolates of *M. bovis* were obtained, and six sheep were noted to have intestinal ulceration. There was no involvement of the lymph nodes draining the hind limb or udder. The potential sources of tuberculosis on this farm were tuberculous cattle, all of which had been removed some years previously, and the surrounding possum population which was known to be tuberculous.

In an attempt to allay fears about the general applicability of the findings reported above to the rest of New Zealand, Allen (1988) presented the results of a sheep slaughter house survey for tuberculous lesions, involving 30 abattoirs, and the inspection of over 10 million sheep killed between 1985 and 1987. No details were given as to how lesions were selected by the meat inspectors, and only 35 samples of potentially tuberculous lesions were subjected to histopathology, two of which were suggestive of tuberculosis. An unstated number were cultured, all with negative findings. This seems to have been a rather inadequate submission rate given that CLA is common in sheep in New Zealand (Nuttall, 1988). Only 9% of the sheep killed in the abattoirs were over 1 year of age. Sheep with the greatest risk of exposure to tuberculosis were thus under-represented in the sample. In this report the number of CLA cases found in a selection of eight abattoirs was also compared on the basis of proximity to tuberculosis endemic areas. A significant

positive association between CLA cases and non-endemic areas was found, which suggested that tuberculosis cases were not being misdiagnosed as CLA. However, the assumption was made that animals from non-endemic areas were only slaughtered in abattoirs in non-endemic areas, and vice versa. This assumption cannot form a reasonable basis for these comparisons, as it is well known that sheep are often slaughtered well outside their district of origin. Furthermore there are many other factors, known and unknown, which affect the distribution of CLA cases across New Zealand.

The disease reports in New Zealand sheep are at variance with historical accounts not only by the comparatively high within-flock prevalence reported, but also in the sites affected. There is a high prevalence of apparently orally acquired and generalised infection in the New Zealand cases. Taken together, this suggests that the epidemiology of the disease is different from that normally found in other countries where endemic tuberculosis has been confined to cattle herds. The most plausible explanation for this is likely to be contact between sheep and tuberculous possums. In a series of trials where livestock were faced with sedated possums placed on the pasture (so as simulate terminally tuberculous animals), sheep generally showed little interest (Sauter and Morris, 1995a). However, despite the level of interest being much lower than that shown by cattle and deer, some sheep were shown to make brief contact with the possums. Contrary to the situation in cattle and deer, the level of interest in possums increased with habituation to possum exposure. This suggests that, as with other livestock that some sheep may have infectious contact with possums, but this is likely to be at a much lower frequency than for cattle and deer under similar circumstances. This direct contact could readily explain the high frequency of orally acquired infections in sheep, with the high proportion of generalised cases resulting from a period of gradual and prolonged development of infection in a susceptible host.

Goats (Capra hircus)

Goats are susceptible to infection with *M. bovis*, and have been shown to develop lesions with similar distribution and histopathological appearance to those found in cattle (Francis, 1958). The lesions are typically caseo-calcareous and often involve the lungs and associated lymph nodes. Infection of the udder has been noted in a number of reports, and has been linked to transmission of tuberculosis to offspring

(Francis 1958; Soliman *et al.*, 1958). *Mycobacterium bovis* infection can cause generalised and progressive disease, with signs such as respiratory distress, hoarse cough, weight loss and decreased milk production (Bernabe *et al.*, 1990/91, cited by Cousins *et al.*, 1993).

The prevalence in goats from historical accounts has usually been below 2%, but in one German herd a prevalence of 21% was found where the goats had close contact with tuberculous cattle (Beller, 1942, cited Luke, 1958). In one British dairy herd, 42% of the herd were diagnosed tuberculous by necropsy examination, following slaughter of half of the herd. Transmission in this instance was believed to have been through feeding of goat milk contaminated with *M. bovis*. Although the disease is typically uncommon in goats this does not appear to be due to any innate resistance to infection, as experimental inoculations have shown goats to be as susceptible as calves (Griffith, 1907). Goats have been implicated as the cause of infection in in-contact cattle (Reidy, 1934, cited by Francis 1958; Hutson, 1941, cited by Luke, 1958), and conversely cattle have been implicated as the cause of tuberculosis in in-contact goats (Carmichael, 1938b; Milne, 1955, cited by Francis, 1958; Cousins, *et al.*, 1993)

Bovine tuberculosis is endemic in goats in Spain and some other Mediterranean countries, and its presence may be serious enough to compromise the eradication of tuberculosis from cattle herds (Vidal *et al.*, 1995). Recently, *M. bovis* infection has been studied in 19 heavily infected milking goat herds in the north-east of Spain (O'Reilly and Daborn, 1995). Emaciation was the most common clinical sign, and annual mortality attributable to the disease was up to 50%, thus confirming the susceptibility of the species. Lung lesions with large caseous nodules and cavitation were notable findings on necropsy. Lesions in Spanish goats tend to be more severe, and to contain fewer organisms, than those in cattle (Gutiérrez Cancela and García Marín, 1993). In another Spanish study of 13 goat herds, the prevalence of tuberculin skin test reactivity was found to vary between 1.2 and 57.6%, thus suggesting that intraspecific transmission was common under the systems of husbandry employed (Vidal *et al.*, 1995). The strains of *M. bovis* infecting goats in Spain, have been shown by molecular typing to be similar to each other, but distinctly different to those infecting cattle (Gutiérrez *et al.*, 1995; Liébana *et al.*, 1997), thus suggesting that the disease is goat adapted, and is transmitted

independently of the disease in cattle. This may explain the high prevalence of disease and the severity of clinical signs seen in the Spanish goat herds, which is at odds with the usual historical accounts of *M. bovis* infection in this species.

Infections with *Mycobacterium bovis* in wild goats in New Zealand have been recognised since 1972 (Allen, 1987). In the 1980s in New Zealand many wild goats were captured and used in domestic goat farming operations. During this period tuberculosis was identified in wild goats captured in areas with endemic tuberculosis (Sanson, 1988). From the West Coast the prevalence of reactors to the intradermal tuberculin injections was found to be 7.2%. Individual groups of goats had reactor prevalences as high as 31%. Of those that were necropsied 45.5% had tuberculous lesions. Gross pathology of tissues was similar to that seen in cattle, and usually involved the lungs and nodes of the head and thoracic cavity, but lesions were also found in all major body nodes and some with generalised disease were detected. Lesions contained yellow caseous material and were usually encapsulated. Calcification was not a feature of the disease in these wild goats and no goats with suppurating sinuses have been reported.

Rabbits (*Oryctolagus cuniculus*)

There has been only one reported case of tuberculosis caused by *M. bovis* in a free-living rabbit (Gill and Jackson, 1993). This animal was caught by the dog of a pest destruction worker in Central Otago. Severe lesions were present in the lungs and kidneys, and one prescapular lymph node, which suggested that the infection could possibly have been acquired by a bite from an infected predator.

The rabbit has an extreme susceptibility to the disease, when demonstrated by experimental airborne or parenteral inoculation (Francis, 1958), but appears to have a high degree of natural resistance to infection by the oral route. Severe lesions develop in the kidneys and the lungs, which are typified by caseation, necrosis, numerous bacilli, fibrous encapsulation and epithelioid cell infiltration (Thorns *et al.*, 1982).

Wild rabbits frequently share environments with other tuberculous animals, and the remarkable absence of field cases of disease may be best explained by the careful and timid behaviour of the species, low susceptibility to infection by the oral route,

and perhaps other factors which prevent direct contact between other infected hosts and rabbits.

Despite continued efforts to find tuberculous rabbits in various locations in New Zealand, the finding of only one to date suggests that currently rabbits play no significant role in the epidemiology of the disease.

Hares (*Lepus europaeus occidentalis*)

Naturally infected hares have been reported from several parts of the world. They were first reported from South Africa by Paine and Martinaglia 1928 (cited by Keet *et al.*, 1996). Schliesser (1985) also reported that occasional tuberculous hares were found in Germany prior to eradication of the disease from cattle.

Five cases of *M. bovis* infection of wild hares (*Lepus leporis*) have been reported from Argentina where they are regarded as a major pest (de Kantor *et al.*, 1984, cited by O'Reilly and Daborn, 1995). The isolates came from a sample of 369 animals condemned at packing plants, from an estimated 4 million processed over a period of 34 weeks.

In New Zealand, one of six hares caught in 1992, in a continuing study at Flagstaff Flat on the West Coast was found to have tuberculosis (Cooke *et al.*, 1993). Gross lesions were visible in the mesenteric lymph nodes, liver and kidneys, and there was a proliferative pleuritis. Histologically the lesions were characterised by granuloma formation, caseation and the presence of few AFB. The area from which the hare was obtained contained at the time a low density possum population, but one which had an exceptionally high prevalence of tuberculosis (Coleman *et al.*, 1994a). Another tuberculous hare was trapped in the following year in the same area, but at this time the prevalence of tuberculosis in the possums was considerably lower (Coleman *et al.*, 1994b).

The presence (albeit at low prevalence) of infection in hares living in areas in which the disease is endemic, suggests that hares are far more susceptible to oral infection than rabbits, or that there is some aspect of their behaviour, or interaction with other hosts, which predisposes them to infection.

Ferrets (Mustela putorius furo)

History

Ferrets were first introduced to New Zealand in 1879 to help rabbit control efforts. Many hundreds were imported from Australia and Britain later that century. Thousands were also bred locally, and released, up until 1912. By the turn of the century, after the ferrets had become widespread, they came to be regarded as pests, particularly due to the damage inflicted on wild bird life. Legal protection was removed and control campaigns began in the 1930s (Lavers and Clapperton, 1990). New Zealand now has the largest known population of feral ferrets of any country. These are distributed over most of the North and South Islands, but are scarce down the West coast of the South Island, and they have not yet reached as far as the top of Northland on the North Island. No distribution surveys have been conducted since 1962, so the exact extent of the country currently occupied is unknown (Lavers and Clapperton, 1990).

Ecology

Densities of ferrets are highest in pastoral and rough grassland habitats, where rabbits are abundant. A significant positive association has been shown to exist between the abundance of rabbits and the density of ferrets in Otago (Mills, 1994) (Mills 1994). In these areas, such as Central Otago, densities of up to 5 per km² have been recorded (Moller *et al.*, 1996). High country, dense forest and developed grazing land are not preferred habitats for ferrets, as lagomorphs, the staple food, are not abundant or the environment is inhospitable. In these areas ferrets are scarce.

There is a marked sexual dimorphism in the species, with males (1.0-1.6 kg) being much larger than females (0.5-0.9 kg). Intra-sexual territoriality is displayed in this species, such that males and females coexist with overlapping territories, but members of the same sex are excluded from the individual's home range. Male home ranges are larger than females, and may sometimes cover over 300 ha (Moller *et al.*, 1996). Movements of up to 2 km per night have been recorded, and dispersal distances of 50 km in less than 4 months have also been noted (Mills, 1994).

Most breeding takes place in the spring, with litters of 4-8 being born in October to December. Occasional births may occur later in some seasons but the survival of young is thought to be poor (Mills, 1994). Weaning is usually completed by 6-8

weeks, and the young disperse from the natal territory at about 3 months of age (Moors and Lavers, 1981).

The average life span of wild male polecats (the wild ancestor of ferrets) in Britain has been estimated at only 8.1 months (Blandford, 1987). The situation in New Zealand is probably similar, as G. Norbury (pers. comm.), during a large radio-tracking study, has estimated the annual population turnover in Central Otago at around 50%, even on properties with an ample supply of rabbits. A large mortality rate in newly weaned ferrets, through maladjustment to an independent existence, is also expected to be an important cause of natural mortality, but there is no information available on the natural causes of death in wild ferrets.

Ferrets live a solitary existence for most of the time, except when rearing litters and mating. The mating ritual itself is rather traumatic for the female who is grasped on the neck, bitten by the male and dragged around, sometimes for up to 3 hours (Fox, 1988; Besch-Williford, 1987). Males are more aggressive in the breeding season, and become involved in fights. G. Norbury (pers. comm.) has noticed that up to 30% of ferrets have had bite wounds during the breeding season, with the vast majority of these in males, and occurring over the rump and neck area.

Diet

Ferrets staple prey appears to be rabbits in most environments.. Rodents are also commonly eaten, as are many other items when available. These additional foods include; small birds, eggs, lizards, hedgehogs, frogs, eels and various invertebrates (Lavers and Clapperton, 1990). They are strict carnivores, as plant material appears only to be accidentally ingested with other food.

Possoms and hedgehogs, both species which can be infected with *M. bovis*, have apparently been commonly eaten in several areas. Roser and Lavers (1976) found that possum and hedgehog remains appeared in 10.8 and 8.4% of ferret scats respectively, in a Manawatu study. Smith *et al.* (1995) also reported a 4.3% occurrence of both possum and hedgehog in ferret gut contents in Otago and Southland. Robertson (1976) found a 14.5% occurrence of possum, and a 5.5% occurrence of hedgehogs also in gut contents on the Otago Peninsula. Several other studies cited by Smith *et al.* (1995), however failed to show possum as a component

of the diet, and hedgehogs featured less commonly than in the other studies mentioned previously.

Carrion is also eaten, often detected by finding dipteran larvae (maggots) in the scats or stomach contents. The carrion will not always have come from animals found dead, they may have been killed by the ferret, cached, and eaten later. Possum is probably only consumed as carrion (fresh or putrid) as they are likely to be too difficult to kill. Roser and Lavers (1976) found a prevalence of maggots in scats of 13.3%, showing that carrion consumption was common around Pukepuke Lagoon. Mills (1994) also found invertebrates, mostly maggots, in 32% of scats examined from animals in the Otago area. Ferrets have also been seen scavenging in offal pits and on the carcasses of dead stock, and this probably explains why the remains of sheep were noted by Smith *et al.* (1995) in the stomachs of 3 ferrets.

The quantity of certain prey species consumed varies seasonally, and in relation to abundance. Rabbits are most frequently eaten in spring and summer when large numbers of kittens are available. Hedgehogs are also most commonly eaten in the autumn when their populations are at a peak and many of the preferred smaller juveniles are also available (Roser and Lavers, 1976). As there is commonly a peak in deaths of possums in the winter and early spring months (Van den Oord *et al.*, 1995) their availability as carrion should be highest then, and this is reflected in the findings of Roser and Lavers (1976) who observed a higher occurrence of possum in the diet from July to September.

Sex differences in diet are also apparent. Females tend to eat more smaller items, such as frogs, lizards invertebrates and mice; and males the larger prey, such as possums, rabbits and hedgehogs. This trend has been apparent in a number of diet studies of ferrets, and although the differences are subtle and not significant individually, probably represents a true sex difference in diet selection (Smith *et al.*) (Smith and others 1995). The observation that males eat more possums indicates that they are more likely to eat carrion than females. This same phenomenon occurs in stoats, where males have been shown to eat more carrion than females in several studies (King and Moody, 1982; Murphy and Dowding, 1994; Murphy and Dowding, 1995). No diet studies have reported the occurrence of ferret remains in the diet, but cannibalism is known to occur amongst ferrets, particularly of young in the nest by the mother.

Chronicle of *M. bovis* infection in ferrets of New Zealand

Tuberculosis was first reported in ferrets captured on the West Coast in the early 1970s (Stockdale, 1975). The disease was subsequently confirmed by the isolation of *M. bovis* from a ferret in the Taumarunui area, in 1982 (de Lisle *et al.*, 1993). These early reports did not generate much interest in the species as a host of tuberculosis, as it was believed that ferrets were present at only low density in the endemic areas. It was considered that they were simply spillover hosts in areas known to harbour substantial numbers of other tuberculous animals, especially possums, but also deer and pigs. Further isolations of *M. bovis* were not made until 1989 (de Lisle *et al.*, 1993). Between 1989 and 1993 there were only 25 isolates of *M. bovis* obtained from wild ferrets, with 18 of these coming from the MacKenzie Basin, a special tuberculosis investigation area. The other isolates were from ferrets in recognised endemic areas, except for one isolate from near Otaki in the Horowhenua.

Mycobacterium bovis was also isolated from ferrets from five fitch farms from both the North and South Islands prior to 1985 (Anon, 1984). Feeding infected offal from Game Packing Houses (Anon, 1982), or carcasses of infected possums was believed to have been the cause of tuberculosis on these fitch farms. Farmed mink in Europe and North America have also become infected with *M. bovis* as a result of being fed uncooked offal from domestic stock in areas where tuberculosis was common (Symmers *et al.*, 1953).

After 1992 there was sufficient interest generated by the tuberculous ferrets so far identified that the number of examinations and samples from these animals escalated dramatically. This was due to the suggestion from various sources that ferrets as well as possums, may be responsible for infecting cattle and deer. This belief was given its first support by the MacKenzie Basin wildlife studies from 1990 to 1992 (Walker, *et al.*, 1993) which found an association between the presence of infected ferrets and properties with reactor cattle, on which there were considered to be few or no possums.

Between March and June 1993, five areas (Reporoa, Waitarere Beach, Wairio, Amuri and Hakataramea), were surveyed for the presence of tuberculosis in predators (Cowan, 1994). These areas were selected as it was believed that wildlife other than possums may have been responsible for the origin, maintenance and

recurrence of tuberculosis infection in livestock. Tuberculosis was diagnosed in ferrets from Wairio, Amuri and Hakataramea only, but not from any possums captured before or during the trapping exercise on those properties. These findings increased the speculation as to whether tuberculous ferrets could be responsible for infecting cattle.

Possum control operations in some endemic localities were also failing to achieve effective reduction of the reactor incidence in areas where tuberculous ferrets had been identified (Atkinson and Cowan, 1994; Mackenzie and de Lisle 1996).

This scenario of infected ferrets being identified in areas in which possums are scarce or in which few or no tuberculous possums have been found has continued up to the present. The North Canterbury area is one such area in which the number of herds in which evidence of *M. bovis* infection has been found, continues to rise. In this area it has been inferred that the apparent front of infection is spreading faster than traditionally seen where the possum has been implicated as the principal host (Livingstone, 1996). Local opinion is that the infection has apparently been spreading over watersheds rather than along them as is usually the case with infected possum host expansion (Livingstone, 1996). Ferrets are viewed locally as the principal cause of the continued expansion of the North Canterbury endemic area as possums are reported to exist at only low density over much of the area and only 0.56% of 356 possums examined have been found tuberculous in local investigations.

The Central Otago endemic area is another in which ferrets have been proposed as the cause of cattle and deer infections. This is an area in which possum abundance is reported to be comparatively low (Ragg *et al.*, 1995b) (but high in patches), and in which the density of ferrets is high, being supported by large populations of rabbits. Tuberculous ferrets are widespread and in this area. Small numbers of tuberculous ferrets have been found during 1996 in coastal Manawatu in surveys subsequent to a spate of herd breakdowns, (H. Benard pers. comm.). Here again the ferrets have been suggested as the cause of the associated herd breakdowns, due to the inability to find infected possums on and around the farms with the reactor cattle, during cross-sectional sampling.

Association with tuberculosis in livestock

In the MacKenzie Basin survey (Walker *et al.*, 1993), 13 tuberculous ferrets were recovered from properties which had suffered infection in livestock in the previous 2 years. No infected carnivores were found on properties with uninfected cattle or deer. This association was statistically significant.

In the three surveys which found infected ferrets, reported by Cowan (1994), all cases of tuberculous predators, principally ferrets, came from properties on which cattle reactors had been detected.

In a predator study of 21 properties in Otago and Southland Ragg *et al.* (1995b) found 11 of 17 properties with infected livestock also carried infected ferrets. Significantly more ferrets were infected with tuberculosis on properties with infected cattle than properties without infected stock. There was however no significant difference between ferret abundance on the infected and uninfected properties. No significant association was found between properties that had a low or high incidence of tuberculosis in cattle and the prevalence of tuberculosis in the ferrets, or the abundance of tuberculous ferrets, on the same properties. There was however a significant association found between the ratio of rabbit abundance to ferret abundance, and the prevalence of tuberculosis in the ferrets. This is probably due to the spring-early summer population peak in the rabbits, coinciding with the time of peak prevalence in older ferrets, prior to the recruitment of disease free offspring. It is extremely unlikely that ferrets will contract tuberculosis from eating rabbits, as the disease in wild rabbits is extremely rare (Gill and Jackson, 1993).

In recent studies in the Pigroot area of Otago (Ragg and Walker, 1996), both possums (n = 1202) and ferrets (n = 161) were trapped and examined from both the northern and southern sides of the Kakanui/Horse range. The only tuberculous animals identified were 10 ferrets, and these all came from the southern side of the range which had been experiencing tuberculosis breakdowns in cattle and deer herds. The northern side of the range was considered to be a tuberculosis free area.

Although infected ferrets have not been found in areas without evidence of infection in livestock, the same cannot be said on a smaller geographical scale. Tuberculous ferrets are often captured in endemic areas (e.g. North Canterbury) from properties on which there has been no history of tuberculosis in susceptible livestock.

DNA fingerprinting using restriction endonuclease analysis (REA) has been used extensively in New Zealand to identify the source, and the relationship between *M. bovis* isolates from outbreaks of tuberculosis in domestic stock. One hundred and twenty five isolates from the MacKenzie Basin outbreaks were REA typed (de Lisle *et al.*, 1995). From the results it was deduced that there were at least two separate introductions of *M. bovis* to the area, and that although mutations had occurred, the same basic types were involved in both domestic stock and wildlife, including ferrets, in the same area. REA data from Table Hill, Milton (Ragg *et al.* 1995a) has also shown that there were two closely related REA types present in wildlife and that one of these had also been present in domestic deer. Atkinson and Cowan (1994) REA typed three ferret and one cat isolate from animals captured adjacent to Lake Wairarapa. The REA types (or closely related ones) had been previously seen in a variety of other local species, including possums, cattle, and deer. There have been no reports of ferret and domestic stock isolates of *M. bovis*, from the same locality, having distinctly different REA types (D. Collins pers. comm.).

Interactions with livestock

Sauter and Morris (1995a) have shown that ferrets sedated, so as to simulate the behaviour of a sick or moribund animal suffering from advanced tuberculosis, attract the interest of both cattle and deer. The curiosity of the stock brought some animals from a considerable distance to closely investigate the sedated ferret. This usually involved only observing and sniffing from a distance, but a significant amount of time was spent by some animals within a distance of 1.5 metres, and a small amount of time was spent in actual physical contact with the ferret. The degree of interest, and the amount of time spent close to or touching the ferret was far less than that spent with possums under similar circumstances. It is likely that these ruminants find the smell of ferrets unattractive, as some mustelid secretions are actually used as herbivore repellents (Sullivan *et al.*, 1990). However, despite the low probability of deer-ferret interactions occurring, there is one report of a group of yearling deer, seen harassing and stomping a sickly tuberculous ferret on a Central Otago property (Boyd, 1996).

Ferrets that are threatened will hiss, scream and bite if not handled carefully. It is quite likely, that although the close investigation of a sick (or dead) tuberculous ferrets by stock will be a rare event, that deer or cattle may become infected with *M.*

bovis after being bitten, or inhaling an infectious aerosol, or by ingesting bacilli after licking a ferret with mycobacterial contamination of the skin. G. Norbury (pers. comm.) has noted that over 60% of dead radio-collared ferrets died above the surface, thus ensuring that the majority of moribund animals are potentially available for investigation by curious stock.

Prevalence of tuberculosis

A number of cross sectional studies have been carried out to determine if tuberculous ferrets have been present in particular areas, and to provide estimates of the disease prevalence. These are recorded in Table 2-II below. In all of these studies the prevalence has been determined initially by recognition of gross lesions at necropsy with later confirmation by histopathology or mycobacteriology.

Table 2-II. Prevalence of tuberculosis in ferrets identified by gross lesions at necropsy

Investigation area	Prevalence (95% CI)	Source of data
MacKenzie Basin	15.5 (8.5-25.1)	Walker <i>et al.</i> , 1993
Wairio, Wairarapa	10.2 (3.4 - 22.2)	Cowan, 1994
Amuri, North Canterbury	7.9 (1.6 - 17.8)	Cowan, 1994
Hakataramea Valley, adjacent MacKenzie Basin	1.7 (0.5 - 8.7)	Cowan, 1994
Otago and Southland	17.9(14.9 - 21.5)	Ragg <i>et al.</i> , 1995b
Scargill, North Canterbury	22.2 (13.8 - 38.5)	Caley, 1995
Pigroot, East Otago	6.8 (2.7 - 10.8)	Ragg and Walker, 1996
North Canterbury	14.0 (11.5 - 16.5)	Oliver, 1996
Tiromoana, North Canterbury	10.5 (3.8 - 16.7)	Caley, 1995

As only animals with gross lesions were used to determine the prevalence in the above studies, it is certain that additional infected ferrets with no sign of lesions at necropsy will have been overlooked. The likelihood of this occurring may be similar to estimates in badgers, where it is believed, that only two thirds of infected animals will have recognisable lesions at necropsy (Gallagher *et al.*, 1976). The

New Zealand studies may have suffered a loss of diagnostic sensitivity in this regard as many of the necropsies have been conducted by non-veterinarians, working without prior guidelines on the appearance of the gross pathology of tuberculosis in ferrets, or the value of various forms of examination. Extrapolation from the gross pathology of possums is a very poor guide to diagnosis.

In the MacKenzie Basin survey there was no significant difference in the prevalence found between males (9/78) and females (4/59), nor between immature (5/47) and mature (8/90) animals. These results may be somewhat confounded by the inclusion of animals not at risk, because they were captured from the tuberculosis clear area. Ragg *et al.* (1995a) found a significant difference between the prevalence in juvenile ferrets (2.5%) and adults (22.4%) caught between January and April, when the age distinction could be made with certainty. This age-linked effect continued into adulthood, as there was a significant rise in adult tuberculosis prevalence (to 32.2%) between winter and summer. In the same study, male ferrets were shown to have a significantly higher prevalence of tuberculosis (14.8%) than females (9.5%).

The moderate, but presumably underestimated prevalence of the disease shown in the above studies, and the trend for the prevalence to rise with age, suggests that contact with the organism is common and occurs at a steady rate throughout the life of the ferret. This is consistent with the disease being principally acquired by scavenging, and demonstrates that there may indeed be a small, but significant, pool of dead and dying tuberculous ferrets found in some areas, particularly those with abundant ferrets.

Pathology of *M. bovis* infection

Traditionally the ferret has been regarded as highly susceptible to infection with a range of mycobacteria, including avian, human and bovine tuberculosis (Fox, 1988). There is a shortage adequate reports on the occurrence of mycobacterial infections in ferrets in the literature despite such infections having been commonly encountered in the past. Apparently up to 60% of ferrets obtained from dealers in Britain were infected with *M. avium*, presumed caused by the common practice of feeding sick poultry to ferrets (Iland *et al.*, 1951). Most of the early accounts of tuberculosis in ferrets pertain to animals kept for research in Europe and the United Kingdom between 1929 and 1953. These investigations have shown in fact that the

ferret is highly susceptible only to *M. bovis* infection, and more resistant to infection by *M. avium* and *M. tuberculosis* (Dunkin *et al.*, 1929; Iland *et al.*, 1951). There have been more recent accounts of the lesions in tuberculous ferrets generated from necropsies during cross sectional studies in New Zealand (Walker *et al.*, 1993; Ragg *et al.*, 1995c). All the detailed accounts of *M. bovis* infection however pertain only to advanced cases, and as such the accepted view of the pathology has tended to be somewhat biased by the end-stage disease observed.

The first comprehensive account of *M. bovis* pathology in the ferret was given by Dunkin *et al.* (1929). In this report three ferrets were presumed infected with *M. bovis*, probably contracted by drinking raw cows' milk, although the bovine type bacillus was isolated from only one animal. All were necropsied in the terminal stages of the disease. Characteristic mesenteric lymph node lesions and pulmonary tubercles were present in each case, and hepatic and splenic lesions were present in two. Histologically the cases were characterised by enormous numbers of AFBs in gross lesions and by the widespread nature of the disease. Two ferrets were also experimentally infected with *M. bovis*, one animal by a 1mg subcutaneous dose, and the other by the same dose orally. The ferret dosed parenterally developed a 25 mm lesion at the injection site and died from generalised tuberculosis 7 weeks post inoculation. The other animal with the oral infection was killed at 18 weeks and showed caseation of the mesenteric lymph node and signs of early generalised infection.

The next comprehensive report on the lesions of tuberculous ferrets appeared in 1951 (Iland *et al.*, 1951) but was more fully reported 2 years later (Symmers *et al.*, 1953). In this paper, one apparently healthy female ferret from a newly received batch of experimental animals sickened and was euthanased when paralysed and in extremis, after a period of only 5 days. At necropsy the animal was found to be suffering from a tuberculous pleurisy and peritonitis caused by *M. bovis*. The abdominal lymph nodes were enlarged and undergoing coagulative necrosis. Lesions contained enormous numbers of intracellular AFBs. Pulmonary nodules were present but were believed to be pre-existing lipid granulomas secondarily infected with tubercle bacilli. Adductor muscle degeneration of the hind legs was thought to have been the cause of the hind limb weakness and paralysis.

A mature male ferret was experimentally infected by Symmers *et al.*, (1953), using intramuscular inoculation of approximately 50,000 bacilli isolated from the naturally infected female. This animal remained in apparent good health for 6 months, then suddenly sickened and developed hind limb paralysis. The animal, moribund after five days of illness, was euthanased. The necropsy appearance was characterised by gross enlargement of the spleen, liver, and abdominal lymph nodes. Histopathology demonstrated that the enlargement of the liver and spleen was caused principally by macrophage accumulation, but this was associated with far fewer AFBs than in the natural case. Lymph node enlargement was attributed principally to lymphoid hyperplasia. Bone marrow was largely replaced by macrophages, and the lungs contained lesions similar to the natural case. There were no necrotic lesions in this inoculated animal.

An experimental intraperitoneal inoculation of several thousand *M. bovis* organisms in eight 10 week old ferrets (Thorns *et al.*, 1982) produced miliary and diffuse lesions composed of lymphocytic infiltrates, 6 weeks post inoculation. Large numbers of AFB in the lung, liver, spleen and mesenteric lymph node, were associated with necrosis of inflammatory cells.

More recently Ragg *et al.*. (1995c) has given a detailed account of the gross lesion distribution in 94 ferrets infected with *M. bovis*. These were mostly collected by trapping on farmland in the Otago and Southland areas. Single site lesions were identified in 56.4% of the ferrets, and 19.1% had three or more sites involved. The mesenteric lymph node was the most common site of infection (34.5% of all lesions), with the retropharyngeal (17%) and the caudal cervical lymph nodes (16.4%) also frequently involved. Only 2.9% of the identified tuberculous lesions were in the lungs.

Of the single-site lesions, 60.4% were in the mesenteric lymph node, indicating, as with the earlier reports on ferret tuberculosis, that most infection appears to be associated with the gut, and ingestion of tuberculous carrion or prey. Peripheral lymph node lesion sites accounted for 25.5% of all lesions, which may imply that intraspecific transmission by bite wounding also occurs.

Gross lesion sites were also recorded by Walker *et al.* (1993) in ferrets obtained from the MacKenzie Basin. Five ferrets had lesions in the caudal cervical lymph

nodes, three in mandibular lymph nodes, two in mesenteric nodes and one case each in the axillary, retropharyngeal and bronchial lymph nodes. Lung lesions were identified in four cases. The lesion distribution identified does not correlate well with the more detailed study of Ragg *et al.* (1995c), where the mesenteric node was identified as the most commonly diseased site. It can be reasonably assumed that the study suffered from the absence of any adequate description of the expected gross pathology and possibly the limited experience of the operators with examination of ferrets.

Routes of *M. bovis* excretion

There has been little reported on the routes by which ferrets might possibly excrete bacilli once infected. Walker *et al.* (1993) isolated *M. bovis* from a pharyngeal swab from a ferret with a retropharyngeal lymph node lesion. Three ferrets with extensive gross lesions collected during the Amuri survey (Cowan 1994) had *M. bovis* isolated from both rectal and tracheal swabs. Swabs from ferrets with single gross retropharyngeal or mesenteric lymph node lesions failed to grow *M. bovis*.

In the natural and experimental *M. bovis* infections of ferrets, described by Dunkin *et al.* (1929) and Symmers *et al.* (1953) all ferrets had liver lesions and one was noted to have mucosal tubercles in the gut. Lesions of both these sites may shed bacilli into the intestinal tract, and may account for the faecal isolations reported above. Lung lesions reported in ferrets may also excrete bacilli into the airways and may account for the isolates from tracheal swabs and the pharynx. Pharyngeal isolates may also come from secretions in the oral cavity or directly from the oropharyngeal tonsillar epithelium which has been shown to shed *M. bovis* in other species (Nugent and Lugton 1995).

Ragg *et al.* (1995c) also found 6/94 infected ferrets had draining tuberculous fistulae connected to underlying superficial lymph nodes. These will certainly be responsible for shedding bacilli onto the skin surface of the ferret, and into the environment. Concentrations of bacilli would be expected in their regularly used den sites, although the organisms may persist for only a few weeks even in favoured sites (Jackson *et al.*, 1995).

Immunology of *M. bovis* infection

Ferrets are capable of mounting delayed type hypersensitivity responses to PPD, after either stimulation with Freund's complete adjuvant (Kauffman, 1981), or *M. bovis* inoculation (Thorns *et al.*, 1982). These responses however are slow by comparison with rabbits and guinea pigs (Thorns *et al.*, 1982), being first demonstrated at 36 days post inoculation. They were not demonstrated in all ferrets inoculated with Freund's adjuvant. The associated cellular infiltrate of the skin is monocytic in nature, and without sign of necrosis.

Lymphocyte transformation assays have demonstrated that ferret lymphocytes are capable of responding to a variety of mitogens, including; phytohaemagglutinin (PHA), concanavalin A, poke weed mitogen, and streptokinase (Kauffman, 1981; Thorns *et al.*, 1982). The response to PHA however was depressed following experimental infection with *M. bovis*, and lymphocytes failed to show mitogenic responses after the addition of PPD to the cell cultures (Thorns *et al.*, 1982).

Antibody responses to *M. bovis* infection have been detected in ferrets (Thorns *et al.*, 1982; Chin *et al.*, 1995). Thorns *et al.* (1982) showed that responses measured by complement fixation test or passive haemagglutination test only occurred in 3 of 6 ferrets, commencing 2 weeks post inoculation. Similarly the ELISA responses detected by Chin *et al.* (1995) were only apparent in 65% of infected ferrets.

The histological appearance of advanced tuberculous lesions in the genus *Mustela* is characterised by macrophage accumulation, large numbers of AFB, especially at the periphery of necrotic centres, with many macrophages containing several bacilli. Giant cells are uncommon, and caseation and fibrosis rare (Dunkin *et al.* 1929; Head, 1959; Adamesteanu *et al.*, 1970; Thorns *et al.*, 1982). Large numbers of *M. bovis* have been found to be toxic for ferret lymphocytes in vitro (Thorns *et al.*, 1982).

It appears that the response to infection by *M. bovis* is not as aggressive and destructive of tissue as it is in many species in which the lesions are often caseous in nature. The development of the cell mediated response appears to be slow, and is possibly compromised by the presence *M. bovis* antigens, which are capable of suppressing lymphocyte reactivity. High numbers of bacilli are capable of inducing local cell necrosis.

Conclusions

On the available evidence an association between the presence of tuberculosis in ferrets and livestock has been established, although it remains uncertain whether this relationship is causal. The association exists only on a broad scale and seems to be independent of the abundance of ferrets, infected or otherwise. Ferrets, other wildlife and stock in the same area appear to share the same or very closely related *M. bovis* REA types. A possible interpretation of the field evidence is that the ferrets, probably in conjunction with other wildlife have been directly transmitting infection to the stock in the same area. An alternative interpretation is that the ferrets and stock have both been infected by a common source such as possums. As yet there is insufficient evidence to assess the validity of these alternative explanations.

Although it has been shown that ferrets are capable of being infected with *M. bovis* and often die from the disease, it is still uncertain as to whether they can transmit infection amongst themselves, either pseudo-vertically or horizontally, at a sufficient rate to maintain the disease in the ferret population without regular reintroduction by cross-infection from other species.

Current evidence suggests that ferrets are capable of infecting cattle and deer, although this is likely to occur uncommonly. Further investigation of this issue needs to be pursued by experimental studies. The other important dilemma facing authorities, is whether the ferret should be regarded as a feral reservoir/maintenance host, or simply a spill-over host of *M. bovis*. Currently there is insufficient information available on the diagnosis, susceptibility, pathogenesis and epidemiology of the disease in ferrets, thus necessitating additional research, before any definitive pronouncements on the host status of the ferret can be made. However, developments in this area are required urgently so that sound species management strategies can be designed.

Stoats (Mustela erminea)

Ecology

Stoats were first introduced from Britain in 1884 to assist in controlling the rabbit, which had after introduction soon established itself as a pest. Stoats rapidly colonised all of the North and South Island, and other islands close offshore. They will live in any habitat in which they can find prey, from the coastal beaches

through to the alpine high country above the tree line. In open country they are less common than ferrets, but in forests more numerous.

The most commonly eaten foods are birds, mice, rats, lagomorphs, possums, and insects. Possums were found to comprise 11.4% of the diet (by weight) in 1250 stoat guts examined from various parts of New Zealand (King and Moody, 1982). Items eaten less frequently include; hedgehog, lizards, crayfish, eel, carrion and rubbish (King, 1990). Male stoats are known to eat more possums and carrion than females (King and Moody, 1982).

Possum remains were found to occur in 20% and 29%, of gut and scat samples respectively in males, with no evidence of possum consumption detected in females (Murphy and Dowding, 1994). Similarly, possum was found in the gut content of 4 of 65 male stoats, but none of 31 females examined, and was also found in 21% of 33 scats from known males, but in none of 48 scats from females examined by Murphy and Dowding (1995). It is unlikely that possums are killed by male stoats, as they would be too formidable a prey, but Murphy and Dowding (1994) found that male stoats were more likely to frequent roadsides than females, and also more likely to scavenge road-killed possums. The carrion from trappers, hunters, road casualties and natural deaths is eaten fresh, but is avoided when putrefying and fly-blown (King and Moody, 1982). Maggots have only been reported in gut contents of one of 65 male stoats, and in one of 33 male scats examined by Murphy and Dowding (1995). Evidence of cannibalism was found in four of 1514 stoat gut samples examined by King and Moody (1982), and a nestling stoat was found in the gut of one of 65 male stoats by Murphy and Dowding (1995).

Breeding occurs in September through to November, with up to 13 young being born. Female offspring are sexually precocious, and are mated when 3 - 5 weeks old. Fertilisation is followed by delayed blastocyst implantation in all females. Dispersal of young has usually finished by early February, when the offspring are 6 - 8 weeks old. Juvenile males have been known to disperse up to 23 km within weeks of leaving litter mates (King and McMillan, 1982), and Murphy and Dowding (1995) observed one female stoat which travelled at least 65 km within 4 weeks.

Stoats are generally solitary animals for most of the year. Opposite sexes have overlapping home ranges, but animals defend their home ranges against members of the same sex. Home ranges of stoats in New Zealand forests are reasonably large, and estimates ranged between 20 and 368 ha for individuals in a study in beech forest by Murphy and Dowding (1994).

The populations of stoats are unstable and subject to wide fluctuation dependent upon food supplies. Because of the difficulty of trapping stoats, population estimates are difficult to make, but where they have been trapped in association with ferrets, the numbers of stoats have been fewer, typically one order of magnitude lower, than for ferrets in the same area (Walker *et al.*, 1993; Cowan, 1994; Ragg *et al.*, 1995b; Caley, 1995b). The life span is short, and there are highly variable rates of birth and death. In New Zealand most stoats are less than 2 years old, but a few individuals may reach ages of 6 - 8 years (King, 1990).

Disease in stoats

Tuberculosis in stoats seems to have been first recorded by Coleman (1975) in animals trapped as a bycatch in a possum investigation in the Hohonu Ranges of the South Island. No details were provided with these early cases, but since that time predators have been specifically targeted in several studies.

Walker *et al.*, (1993) examined one necropsy-negative stoat from an area in which 15.5% of 84 ferrets were found to be infected. In a series of five cross-sectional studies reported by Cowan (1994), nine stoats were captured, but none were suspected of being tuberculous at necropsy examination. Three of these stoats (one each), came from three areas where tuberculosis had been identified at necropsy in ferrets, the prevalence in ferrets ranging from 1.7% to 10.2%. Ragg *et al.*, (1995b) found one of 61 stoats collected in Central Otago with tuberculous lesions at necropsy. Disease in this animal was confirmed by culture. Of 548 ferrets captured from the same area, 17.9% were found to be infected with tuberculosis. Coleman *et al.*, (1994a) found two of two mature male stoats captured on the West Coast in 1992 infected with tuberculosis. These cases came from an environment where the prevalence of tuberculosis in possums was exceptionally high, and estimated to be 60%. The following year (1993) a single stoat trapped in the same area was found to be infected with *M. bovis* (Coleman *et al.*, 1994b). The prevalence of disease in 1993, amongst the possum population was found to be 16.7%. The following year

(1994), at the same site, none of a further two stoats examined showed evidence of tuberculous lesions (Coleman and Cooke, 1995). The apparent prevalence of tuberculosis in the possums at this time was lower at 7.8%.

From what little data there is available on the disease in stoats, it seems that they are capable of becoming infected, and in some instances of developing disease, although apparently less likely to do so than ferrets in the same environment. Due to the preference shown by male stoats for eating possums, it would appear that they may be more at risk than females of becoming infected. Carrion consumption may have been the cause of disease in both stoats examined in the 1992 study reported by Coleman *et al.*, (1994), as the availability of tuberculous possum carrion would have been very high at that time.

Weasels (*Mustela nivalis*)

These mustelids are far less common than either ferrets or stoats in New Zealand. They are small, inconspicuous and rarely seen in the countryside. Although Allen (1991) reported that infected weasels had been found in New Zealand, this claim was not substantiated by specific evidence. There have not been any authenticated published reports of this species being infected with *M. bovis* in any country. As no weasels were examined during the course of this research, and no role for them in the epidemiology of *M. bovis* infection is believed to exist, no further mention will be made of this species.

Cats (*Felis catus*)

Ecology

Feral cats are widespread in New Zealand and are found in all habitat types (Fitzgerald, 1990). Density estimates for the North and South Islands have not been published, but it is likely that several cats are present per square kilometre in most areas. Trap catches for cats seem to approximately parallel those for ferrets in the same environment (Cowan, 1994; Caley, 1995a) but are not closely associated with the abundance of rabbits (Mills, 1994), one of the principal prey species. In areas with high rabbit populations, ferrets appear more numerous than cats, when judged by trap catch data.

Cats are considered to be solitary animals, but with a fairly complex, family-based, social organisation. Home ranges overlap, and within these ranges den sites are

used only temporarily, except during the rearing of litters. One to 10 young are born in each litter, and each female may breed two or three times per year if conditions are satisfactory. Survival of kittens is likely to be poor, and adults are unlikely to live beyond 6 years of age.

The majority of the diet consists of mammals, principally rabbits, rodents, hares and possums. Some of the diet is eaten as carrion, particularly possum, which was estimated to comprise 17.6% by weight of the diet of cats living in the Orongorongo Valley (Fitzgerald and Karl, 1979), and 12.6% by weight of the diet of cats in a farming area in Hawke's Bay (Langham, 1990). This possum consumption occurred in most seasons but was most prevalent in the spring when young backriders, becoming independent, were preyed upon, and also in the winter when dead adults were available following periods of inclement weather. Stoats are also eaten by cats, though infrequently (Wodzicki, 1950; Fitzgerald and Karl, 1979).

Disease in cats

Francis (1958), in reviewing the literature on tuberculosis in cats has shown that infections are almost always due to *M. bovis*. However, more recently reports from Britain suggest that infections from a bacillus intermediate in type between *M. tuberculosis* and *M. bovis* may be commonplace (Gunn-Moore *et al.*, 1996; Blunden and Smith, 1996). The source of this novel bacillus is uncertain, but transmission from wild prey is one possibility that has been considered. The most commonly affected sites are the lungs and the mesenteric lymph nodes, both of which are affected in approximately 65% of lesioned animals (Francis, 1958). Lesions in the lymph nodes of the head, especially the mandibular lymph node, were also common, being found in 11% of lesioned animals. Pleurisy or peritonitis also occurred commonly (15% of cases) and were often associated with effusions. The uterus was also involved in 5% of cases and the skin in 7%. The high prevalence of lesions in kidneys, spleen (approximately 20% each) and the occasional occurrence in unusual sites such as the eye indicate that haematogenous dissemination is common. Testicular lesions have also been recorded.

The principal route of infection in most studies appears to have been via the alimentary tract, and traditionally has been presumed to occur mainly from the ingestion of cow's milk contaminated with *M. bovis* (Francis, 1958). The ileo-colic area appears to be the chief predilection site in the intestine, with the initial

infection being first detected in the local lymph nodes (incomplete primary complexes). However, extensive histopathological examination may reveal the presence of granulomas in ileal Peyer's patches (Blunden and Smith, 1996). Only after extensive lung involvement are gross lesions of the bowel recognised, typically ulcerated Peyer's patches. These ulcers have been attributed to continuous bacillary challenge through swallowing of heavily infected respiratory secretions (Jennings, 1949). Airborne infection is also thought to occur in some circumstances where primary pulmonary complexes have been identified. An example of this occurred in an animal house in Australia, where airborne transmission from an index case with a draining mandibular lesion was believed to have infected several cats and a possum (Isaac, 1983) (Isaac and others 1983). Most pulmonary lesions however seem to be secondary to haematogenous spread from primary mesenteric sites (Innes, 1940). Chaussé (1909, 1910 and 1912, cited by Francis, 1958) was able to detect lesions in 4/15 cats fed on infectious material from tuberculous cattle, whereas 8/10 cats exposed to airborne *M. bovis* developed primary thoracic lesions.

Snider(1971), in a review of the literature, summarised the apparent prevalence of feline tuberculosis in a variety of studies. Values reported in these studies ranged between 0.2 and 13%. It is likely that some of the higher figures are overestimates due to a presentation bias in the animals examined, e.g. the 12% prevalence found by Yost (1921, cited by Dobson, 1930) is probably strongly influenced by the inclusion of a large proportion of unhealthy individuals, as all the cats necropsied were for disposal by the Berlin meat destructor.

Lesions in cats are characterised by extensive necrosis, caseation and central liquefaction. Early lesions are comprised of non-specific granulomatous tissue consisting primarily of large macrophages. Lymphocytes may be common and polymorphonuclear leucocytes may be present in moderate numbers, especially around liquefied areas. Lesions do not contain giant cells and calcification is unusual. Healing by fibrosis and hyalinisation may occur, but early dissemination is more usual (Snider, 1971). Lesions in mesenteric lymph nodes frequently appear as simple enlargement with small white foci visible through the capsule (Snider *et al.*, 1971). These granulomatous areas progress to engulf the whole node in a caseous tuberculous process, often with associated fibrosis or adhesions to adjacent loops of bowel (if mesenteric nodes are involved). Lung lesions vary, and range

from scattered 2mm tubercles to large areas of consolidation caused by coalescing granulomas. Miliary pulmonary tuberculosis has also been observed in cats. Large lung lesions may cavitate and allow bacilli-laden exudate to enter the airways (Griffith, 1926). Skin lesions are commonly found and are often the reason for presentation of domestic cats to veterinary practitioners. These cutaneous lesions commonly drain fistulae from underlying liquefied nodal lesions, but may also develop at sites independent of lymph node involvement. These isolated skin lesions are thought to arise from percutaneous inoculation of bacilli from infectious bite wounds or scratches from other cats, or wildlife (Jennings, 1949; de Lisle *et al.*, 1990; Gunn-Moore *et al.*, 1996). Most skin lesions become necrotic and develop into indolent ulcers, but some cutaneous and subcutaneous pyogranulomatous lesions appear to remain closed (de Lisle *et al.*, 1990).

Tuberculous cats in New Zealand

Tuberculosis was first noted in wild cats in the early 1970s on the West Coast endemic area of the South Island (Stockdale, 1975). Cross sectional studies of wild cats and other predators were not begun until the late 1980s, when it was proposed that wildlife, other than possums, may have had a role in maintenance of the disease. The first of these studies involved the necropsy of 110 cats from the MacKenzie Basin. Only one infected cat was found, representing a 2.0% (0.05 - 10.5) prevalence in the 50 'at risk' cats trapped from the area in which reactor stock had been found (Walker *et al.*, 1993). This was much lower than the prevalence of 15.5% seen in ferrets in the same area.

Cowan (1994) when summarising the results of several predator surveys describes two of 47 cats from Amuri with individual lesions of tuberculosis in the mandibular and mesenteric lymph nodes, and one of 48 cats from Wairio with a tuberculous lung lesion. The prevalence estimates in the cats at these sites was 4.2% (0.4 - 14.2) and 2.0% (0.1 - 10.5) respectively, and compared with prevalence estimates of 7.9 and 10.2 % in ferrets at the same sites. All infected predators were trapped on properties with recent histories of infection in cattle.

In a large study in the Otago area, Ragg *et al.* (1995b) found a prevalence in cats of 0.9 % (n = 215, 0.12 - 3.6) and a prevalence in ferrets of 17.9 % (n = 548, 14.9 - 21.5). Once again the infected predators were found only in areas from which reactor stock had been sourced.

Ferrets and cats were also trapped at Hohotaka, in the central North Island endemic area (Caley, 1995b). In this study one of 22 ferrets had gross lesions, but *M. bovis* was subsequently isolated from pooled tissues of six in total. Thirty nine cats were trapped during this same exercise, but all were free of gross lesions. Caley (1995a) also necropsied 56 cats from two sites in North Canterbury. None of these cats had gross tuberculous lesions, whereas the prevalence of tuberculosis, derived from necropsy examination of ferrets trapped in the same area, varied between 10.5 and 22.2%. Cats taken from farms in the North Canterbury area have also been examined by MAF personnel. The prevalence of disease determined by gross lesion detection was 1.3% in 76 cats (Oliver, 1996), whereas ferrets in the same area had a tuberculosis prevalence of 14%.

De Lisle (1993) reported a series of 76 cats from which *M. bovis* was isolated. Seventy three of these were domestic cats, and 69 came from tuberculosis endemic areas. The diagnostic samples came mainly from cats with skin lesions (49%) especially around the head and forequarters, or with peripheral lymphadenopathy (22%), especially of the mandibular node. The presenting signs were similar to a series of 19 cats described by Gunn-Moore *et al.*, (1996) and infected by a previously unknown *M. tuberculosis* complex variant. An earlier account by de Lisle *et al.* (1990) on 57 of the same cats found that those from the Wairarapa area and Otago had the same REA types as those found in possums in the same area, raising the possibility that the cats had become infected either by ingestion of, or by conflicts with, infected wildlife. A similar scenario existed with an outbreak of tuberculosis in one farm's cats reported by Orr and Thompson (1992) where large numbers of possums existed in the area and the cats were also occasionally fed feral deer meat. Contaminated skin wounds and scratches resulting from fights with possums may have been the source of the percutaneous infection. This large number of reported cases in New Zealand with skin lesions, presumably arising from scratches and skin wounds, has probably developed through the need for a definitive diagnosis in cases of non-resolving skin lesions in family pets. There are however, likely to have been a far greater number of inapparent cases in domestic cats over the same period, which remained undiagnosed.

Conclusions

Cats have a greater potential to transmit disease by a larger number of routes than many other species, including ferrets, i.e. oral, respiratory, urinary, venereal, congenital, and via open skin lesions. They appear to be highly susceptible to primary pulmonary infection, but much less so to infection via the alimentary tract. The pulmonary environment appears to favour bacillary multiplication and the development of disease, as is the case in numerous other species. Despite the high susceptibility by the respiratory route, and the multitude of potential excretion sites the cat “is not an easy victim to the disease” (Jennings, 1949), this circumstance arising as the alimentary tract is the major route of infection, and cats are moderately resistant to this mode of transmission.

As with ferrets, there appears to be a broad scale relationship between the occurrence of tuberculosis in feral cats and the presence of tuberculosis in domestic stock. The limited New Zealand studies to date have shown the prevalence in feral cats to be considerably lower than those in ferrets in comparable circumstances, despite cats and ferrets sharing very similar dietary components, and in comparable proportions. Possums and carrion form a substantial part of a wild cat’s food intake, and historically, *M. bovis* infected milk must have been commonly drunk by farm cats, but the low prevalence of tuberculosis in cats, both present and past, convincingly demonstrates that they are at least moderately resistant to the establishment of disease by ingestion of contaminated food and that transmission between cats with subsequent development of disease is uncommon. Some cats seem to be able to contain the disease process by fibrous tissue reaction and eventual resolution of lesions. It has been suggested by De Lisle (1993) that infection by feline leukaemia or immunodeficiency virus may predispose cats to disease progression, but tests for viral infection proved negative in the series of 14 tuberculous cats checked by Gunn-Moore *et al.* (1996) suggesting that an immune system compromised by these viral infections may not be one of the causal factors commonly associated with disease.

Cats are most unlikely to be reservoir hosts of the disease in New Zealand or other countries, as bovine tuberculosis has been successfully eradicated in areas where, historically, there has been a moderate prevalence of disease in this species, associated with infection in cattle. Several European studies have however

implicated infected farm cats associating with cattle, as having reintroduced the disease to herds previously tested free of tuberculosis (Beinhauer,1958; Milbrandt and Roemmele, 1960; Pavlas *et al.*, 1965; Schliesser and Bachmeier,1957). If this were the case these situations are likely to represent only isolated incidents with little importance to eradication efforts overall.

Hedgehogs (Erinaceus europaeus)

Ecology

Hedgehogs were first introduced from Britain to New Zealand in 1870, with more being liberated from importations later in that century. They were initially introduced to remind settlers of their homeland, but later came to be viewed as useful for ridding gardens of slugs and snails. Liberations continued into the early part of the twentieth century, thus ensuring their colonisation of all suitable habitat on both the North and South Islands. Hedgehogs are now abundant throughout lowland districts, especially near the coast, but less numerous in the hills and forested country, and rare in mountainous areas (Brockie, 1990). Their distribution is limited by the availability of dry, well-sited and warm nest sites, which they use during the hibernation period.

As hedgehogs are principally insectivores, but will eat almost any animal substance, particularly invertebrates, including fly maggots (Brockie, 1959), it seems quite likely that in endemic areas they could be exposed to *M. bovis* infection from the investigation of decomposing carcasses, particularly those of possums.

Feeding ranges of hedgehogs, particularly the females, in New Zealand are small (e.g. between 1.9 and 3.6 ha) (Reeve 1994). Population densities are unlikely to rise above 2 per hectare, even in preferred habitats, but local aggregation will occur where preferred food is plentiful (Brockie, 1990). They are largely solitary animals except during brief mating encounters and while rearing young.

Breeding in hedgehogs commences as soon as they emerge from their winter hibernation, usually in September. However pregnancies are usually not found till December, and pregnant females can be found right through till the time of hibernation, with some females bearing two litters per season. North Island hedgehogs usually carry 4 - 7 embryos, but nests contain on average only 2.7 young (Brockie, 1990).

Pigs and ferrets are known to prey on hedgehogs, and their carcasses will also be scavenged by a variety of mammals and birds. In a recent study in Central Otago (Smith *et al.*, 1995), hedgehog remains were found in six of 140 (4.3%) ferret stomachs containing food. An earlier Manawatu investigation found an 8.4% prevalence of hedgehog tissues in ferret scats (Roser and Lavers, 1976).

Tuberculous hedgehogs internationally

The first recorded naturally infected case of tuberculosis in a hedgehog occurred in Britain, when *M. bovis* was recovered from a hedgehog found in Regent's Park, London, in 1932 (Hamerton 1933). This animal died from the disease after 5 weeks confinement in the Regent's Park Zoo. Lung lesions were described by Hamerton as being of the "grey hepatisation" type and contained large numbers of AFB. It was believed that the hedgehog may have become infected through drinking cow's milk contaminated with *M. bovis*. Hamerton (1936) also reported the death of another tuberculous hedgehog after nearly a year's captivity in the zoo. *Mycobacterium bovis* infection was not confirmed but lesions were found in the lungs and intestinal tract. Schliesser (1985) also remarked that occasional cases of bovine tuberculosis were found in hedgehogs in Germany prior to the eradication of this disease.

During the Royal Commission on Tuberculosis, Griffith (1907) subjected sixteen hedgehogs to experimental *M. bovis* infections. Six animals infected parenterally with doses of up to 1 mg of culture, showed a predisposition to caseous lesion development in the local lymph nodes and small non-caseating granulomas forming in the liver, spleen, kidney and lungs. Ten other animals were given doses of up to 10 mg of culture orally. Of these four developed no gross or microscopic lesions. The six remaining showed caseous lesions in the retropharyngeal or cervical lymph nodes (n = 4), or small grey lesions in the lungs (n = 3), one animal developed haemorrhagic intestinal lesions and another had small omental lesions with calcification. Although AFB were seen in some of the mesenteric lymph nodes these were not associated with gross lesions.

Thorns *et al.*, (1982) also experimentally infected five hedgehogs by intraperitoneal injection with a dose of 500-5000 *M. bovis* organisms. When these animals were killed at 40-60 days post-inoculation, no gross lesions were visible, although there were microscopic granulomas in the kidneys containing AFB, necrotic cells,

polymorphonuclear leucocytes and epithelioid cells. Acid fast bacilli were also seen in the lungs and spleen with no associated lesions, and mycobacteria were additionally cultured from the liver. Interestingly, the hedgehogs in this study failed to show any specific measurable immunologic responses to the presence of the mycobacteria. It was proposed that the low body temperature, between 33 and 38 °C, may have limited the pathogenicity of the bacilli, but this seems unlikely, given the severity of the disease in brushtail possums (*Trichosurus vulpecula*) which have an average body temperature of 36.2 °C.

Tuberculous hedgehogs in New Zealand

The hedgehog has not been confirmed as a host for *M. bovis* in New Zealand despite hundreds being captured and examined in many cross-sectional studies of tuberculosis in wildlife. There have however, been several unconfirmed cases of tuberculosis in hedgehogs in this country which have arisen in the course of research or wildlife surveys for tuberculosis. In a post-mortem leptospirosis survey of 155 hedgehogs from the southern part of the North Island in 1957, Brockie (1958) found one animal with pulmonary lesions containing AFB. This may in fact be the earliest record of wildlife tuberculosis in New Zealand, eclipsing the infected feral pigs found in 1964 (Ek Dahl, *et al.*, 1970). Beatson found a case of tuberculosis in a West Coast hedgehog in the early 1970s (Stockdale, 1975), although it is uncertain whether the presence of *M. bovis* was confirmed due to loss of records.

Conclusions

It appears that the hedgehog is a naturally moderately resistant species which can mount an effective cell-mediated immune response to *M. bovis*. The disease can be expected to occur in susceptible individuals in wild populations, where exposure to infected carcasses is possible. Indeed the disease may have already been identified on several occasions in New Zealand, but has remained unconfirmed, as isolation of the organism has not been attempted. The lesions reported in naturally infected animals seem to occur predominantly in the lungs, with possible involvement of the gut, and experimentally, caseous lesions have developed in lymph nodes draining the site of entry of the organism. The fact that the disease has not been confirmed in New Zealand, despite many hundreds having been examined, suggests that the lesions will be of a minor nature, or atypical of the disease in other species, and probably occur at a low prevalence. As hedgehogs are eaten by several other

predators, tuberculosis in this species may provide another source of infection for pigs, ferrets and cats.

Rats (Rattus spp.)

Although ship rats (*Rattus rattus*) are common and widespread in New Zealand and other countries (Innes, 1990), there is no information available regarding their susceptibility to *M. bovis* infection. There have been no reports of any wild ship rats with tuberculosis.

The brown rat (*rattus norvegicus*) from which laboratory rats have been bred, is also common in New Zealand but is generally confined to wetland habitats and areas of human habitation (Moors, 1990).

Brown rats are considered to be resistant to *M. bovis* infection, but have been shown to have bacilli persist in lymphatic tissues for months following infection despite minimal development of gross lesions (Wessels, 1941; Thorns *et al.*, 1982). Necrosis and caseation are not apparent in *M. bovis* infections in rats (Wessels, 1941), and this may be due to the failure to develop classic DTH responses, and the tissue damaging responses which often ensue. Granulomatous reactions following infection, which may be visible as pin-head sized lesions in the lungs, are typified by lymphocytic infiltrates and the presence of activated macrophages (epithelioid cells), which appear to be highly protective (Wessels, 1941; Ratcliffe and Palladino, 1952; Thorns *et al.*, 1982).

There have been few isolations of *M. bovis* from wild rats, with most reports coming from England. Bosworth (1940) reported the isolation of *M. bovis*, after guinea pig inoculation of pooled, ground tissues, from two of 167 rats trapped around farm buildings in England. Neither of the rats (presumably *Rattus norvegicus*) showed evidence of gross lesions.

In Italy 19 of 79 brown rats were found to be infected with *M. bovis*. These had been feeding on the edge of the Naviglio river which received the effluent from a Modena slaughter house (Vöhringer, 1964).

More recently in England, Little *et al.*, (1982) described the isolation of *M. bovis* from 2 of 90 brown rats from a dairy/pig farm in south Dorset, on which *M. bovis* was endemic in badgers and had also been isolated from domestic pigs and cattle.

These rats had no gross lesions, and the organism was isolated from pooled lymph nodes. It was speculated that the rats may have become infected through feeding on undigested grain in badger faeces or by feeding on badger carrion. Although the two infected rats were captured near farm buildings rather than badger setts, it was suggested that feeding forays of up to 1 km, or changes of residence may have explained the infected rats presence near the farm buildings (Wilesmith *et al.*, 1986). In another study in East Sussex (Wilesmith *et al.*, 1986), an area with endemic tuberculosis in badgers, 103 rats were examined and a variety of tissues pooled for culture from each animal. No gross lesions or isolates of *M. bovis* were detected, but it was suggested that the large tissue pools cultured may have diluted any bacilli present and lowered the sensitivity of culture.

In areas with endemic tuberculosis, a low prevalence of infection in wild brown rats could be expected through feeding on carcasses of other tuberculous hosts. However, the detection of lesions at necropsy would seem unlikely. If rats are infected they may provide one source of infection for predators, but because of their well demonstrated resistance to infection, they could never be considered as candidates for reservoir host status, and thus will always have an unimportant role in the epidemiology of tuberculosis.

Mice (*Mus musculus*)

Mice are considered to be innately highly resistant to mycobacterial infection, but despite this, they have been used extensively in experimental models of tuberculosis. After being administered large intraperitoneal or intravenous doses of *M. bovis* or *M. tuberculosis*, mice will usually die within a few weeks (Wilson and Miles, 1964). There have been no reports of natural infection of mice reported, and thus it is unlikely that they will have a role in the epidemiology of disease in wild animal populations.

CHAPTER 3

EPIDEMIOLOGY OF *MYCOBACTERIUM BOVIS* INFECTION IN FERAL FERRETS (*MUSTELA* *FURO*) IN NEW ZEALAND: I. PATHOLOGY AND DIAGNOSIS¹

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Abstract

Necropsies of 228 ferrets captured from eight areas in the North and South Islands provided material for an investigation into the epidemiology of tuberculosis in wild ferrets. Mycobacterial culture of pooled lymph nodes (retropharyngeal, respiratory and jejunal) identified the prevalence of infection to be much higher than that estimated from gross lesions only. Seventy-three of the 228 animals examined (32%) were diagnosed as tuberculous. Fifty-three culture-positive ferrets and 18 seemingly uninfected animals were subjected to detailed histopathological examination. The outcomes of these investigations, including the characteristics of the disease, distribution of lesions, and aids to diagnosis, are presented.

Of the wild carnivores found in New Zealand, the disease persists at high prevalence only in ferrets, and is probably maintained principally by ingestion of tuberculous carrion. The course of the disease may be prolonged in some ferrets, but tuberculosis eventually causes the death of many infected animals. Microscopic hepatic granulomas may be considered pathognomonic of the disease, and have potential to be used as a rapid diagnostic tool in ferrets with no gross lesions.

Introduction

In New Zealand it is well recognised that the major impediment to continued progress in the control of tuberculosis is the re-infection of domestic stock populations by infected wildlife, especially the brushtail possum (*Trichosurus vulpecula*), which is known to be a reservoir host for the disease (Morris and Pfeiffer, 1995). Despite tuberculosis having been found in feral cats (*Felis catus*) and ferrets (*Mustela furo*) in New Zealand since the early 1970s (Stockdale, 1975), the disease and its epidemiology in these predators is poorly understood. Tuberculous ferrets are known to be widespread in New Zealand (de Lisle *et al.*, 1993), but their importance to tuberculosis eradication has been surrounded by controversy since the discovery of infected predators in the Mackenzie Basin, where they were first implicated in the transmission of tuberculosis to farmed stock (Walker *et al.*, 1993). Recently it has also been suggested that ferrets could be an additional reservoir host of the disease (Ragg *et al.*, 1995b). Owing to the important implications of these suggestions, and the absence of any comprehensive reports on the diagnosis, pathogenesis and pathology of the disease in ferrets, a study was initiated to gain a basic understanding of the disease process which would help clarify the contribution of this species to the epidemiology of tuberculosis in New Zealand.

A number of cross-sectional studies examining predators have been conducted in New Zealand, and these have provided estimates of the disease prevalence in ferrets at several (Walker *et al.*, 1993; Cowan, 1994; Caley, 1995a; Ragg *et al.*, 1995b; Ragg and Walker; Oliver, 1996). The average prevalence of gross lesions at necropsy (with later confirmation by histopathology or mycobacteriology) for each study has been found to range from 1.7% to 22.2%. As only animals with gross lesions were used to determine the prevalence in the above studies, it is possible that additional infected ferrets with no visible lesions at necropsy will have been overlooked, and thus the true prevalence will be somewhat higher than reported. The likelihood of this occurring may be similar to estimates in badgers, where it is believed that only two thirds of infected animals will have recognisable lesions at necropsy (Gallagher *et al.*, 1976). These New Zealand studies may have suffered a loss of diagnostic sensitivity in this regard, since standardised necropsy and diagnostic methods had not previously been established.

Traditionally, the ferret has been regarded as highly susceptible to infection with a range of mycobacteria, including avian, human and bovine tuberculosis (Fox, 1988). However, investigators in Europe have shown that the ferret is highly susceptible only to *M. bovis* infection, and is more resistant to infection by *M. avium* and *M. tuberculosis* (Dunkin *et al.*, 1929; Iland *et al.*, 1951).

The first account of the pathology *M. bovis* infection in the ferret was given by Dunkin *et al.*, (1929). In this report three naturally infected ferrets were necropsied in the terminal stages of the disease. Enlarged caseating mesenteric lymph node lesions and “pulmonary tubercles” were present in each case, and hepatic and splenic lesions were present in two. Histologically, the cases were characterised by enormous numbers of acid-fast bacilli (AFB) in gross lesions and by the widespread nature of the disease. Two ferrets were also experimentally infected with *M. bovis*, one by a 1 mg subcutaneous dose, and the other by the same dose orally. The ferret dosed parenterally developed a 25 mm lesion at the injection site and died from generalised tuberculosis 7 weeks after inoculation. The other animal with the oral infection was killed at 18 weeks and showed caseation of the mesenteric lymph node and signs of early generalised infection.

The next comprehensive report on the lesions of tuberculous ferrets appeared in 1953 (Symmers *et al.*, 1953). In this paper, one apparently healthy female ferret suddenly became ill and was destroyed when paralysed, after a 5-day illness. At necropsy the animal was found to be suffering from a tuberculous pleuritis and peritonitis caused by *M. bovis*. The abdominal lymph nodes were enlarged and undergoing coagulative necrosis. Lesions contained enormous numbers of intracellular AFB.

A mature male ferret was experimentally infected by Symmers *et al.* (1953), using an intramuscular inoculation of about 50000 bacilli isolated from the naturally infected female. This animal remained in apparent good health for 6 months, then suddenly sickened and developed hind limb paralysis. The animal, moribund after 5 days of illness, was euthanased. There was gross enlargement of the spleen, liver, and abdominal lymph nodes, but without necrosis being evident. Histopathology demonstrated that the bulk of the lesions were due to lymphoid hyperplasia and accumulation of macrophages.

More recently, Ragg *et al.* (1995c) has given a detailed account of the gross lesion distribution in 94 New Zealand ferrets found infected with *M. bovis*. These were mostly collected by trapping on farmland in the Otago and Southland regions. Single site lesions were identified in 56.4% of the ferrets, and 19.1% had three or more sites involved. The mesenteric lymph node was the most common site of involvement (34.5% of all lesions), with the retropharyngeal (17%) and the caudal cervical (prescapular) lymph nodes (16.4%) also frequently involved. Only 2.9% of the identified tuberculous lesions were in the lungs.

Of the single-site lesions, 60.4% were in the mesenteric lymph node, indicating, as with the earlier reports on ferret tuberculosis, that most infection appears to be associated with the gut, and hence putatively with ingestion of tuberculous carrion or prey. Peripheral lymph node lesion sites accounted for 25.5% of all lesions, which may imply that intraspecific transmission by bite wounding also occurs, although possums are commonly infected in peripheral nodes early in the disease, without skin infection being apparently involved (Jackson *et al.*, 1995b).

Gross lesion sites were also recorded by Walker *et al.* (1993) in ferrets obtained from the Mackenzie Basin. Five ferrets had lesions in the caudal cervical lymph nodes, three in mandibular lymph nodes, two in mesenteric nodes and one case each in the axillary, retropharyngeal and bronchial lymph nodes. Lung lesions were identified in four cases. The lesion distribution identified, however, did not correlate well with the more detailed study of Ragg *et al.* (1995c), where the mesenteric node was recorded as the most commonly diseased site.

Unfortunately, all the available detailed accounts of *M. bovis* infection pertain only to advanced cases, and as such the accepted view of the pathology has tended to be biased by the end-stage disease observed. The lack of balanced and detailed information regarding the disease and its pathology in ferrets also made it important to gather data on the range of disease states encountered, as a basic prerequisite to acquiring insight into the pathogenesis and transmission of this disease.

As this investigation progressed, it became evident that in a large proportion of “early” cases, accurate diagnosis at gross necropsy was impossible. Refined procedures, both gross and histological, were sought whereby diagnostic sensitivity could be improved and the true disease status determined sooner.

Materials and Methods

Two hundred and twenty-seven ferrets were collected from eight areas in the North and South Islands from 1994 to 1996. Most animals were acquired by trapping and killed by a blow to the head and a few were found dead. One animal was examined alive and released. Eighty were frozen prior to submission; the remainder were kept chilled until examined. Details on the area of origin and numbers subjected to extensive histopathological examination are shown in Table 3-I. The ferrets examined all came from areas in which bovine tuberculosis was considered endemic, the animals being conveniently submitted by field researchers and trappers who were already working in the areas sampled.

Table 3-I. Geographical origin of ferrets, their tuberculosis status and number subjected to extensive histopathological examination

Area of origin	Number of ferrets examined	Number believed tuberculous	Number subjected to extensive histopathological exam ^a
North Canterbury	137	35	24
Hohotaka	27	6	6
Eastern Wairarapa	19	18	14
Featherston	18	1	0
Table Hill	14	4	4
Alexandra	6	3	1
Lake Ferry	5	5	5
Manakau	1	1	0

a number of tuberculous ferrets only.

Necropsy and data recording procedures

Data collected on each animal prior to necropsy included: origin and whether this was in an area with *M. bovis* infection recognised in possums; weight (g); total length (mm) and sex. After necropsy, details of any lesions were recorded, as was an opinion of the disease status (infected, suspected, or uninfected), and presence of plaques on the lung surface; state of the thymus; condition of the uterus and mammary glands; body fat reserves; estimated age (months) based upon size of animal, teeth wear, uterine, nipple and thymic condition, and time of year (all ferrets were deemed to have been born at the end of October, unless the size of the animal made this improbable). Before the body was opened, a swab (Transwab, Medical Wire and Equipment Co. Ltd.) was passed into the oral cavity and rotated in the pharynx to gather oral secretions and cellular debris, before storage in transport medium.

At necropsy a 3-4 cm strip of skin was carefully reflected from the ventral body surface, taking care to avoid removing the superficial axillary (cubital) nodes. All major peripheral lymph nodes were inspected and incised. After opening the thorax and abdomen, the internal organs were examined and the lungs palpated. The kidneys were incised, the stomach opened and a visual estimate made of the volume of the contents, and the proportion and type of each item present. The intestine was not opened during the necropsy, nor were any other organs incised unless visibly diseased.

From each animal a pool of lymphatic tissues was collected for mycobacteriological examination. This included a half portion (including any diseased sections) of both retropharyngeal lymph nodes, one of each of the bronchial and cranial mediastinal lymph nodes, and about half the jejunal (mesenteric) lymph node (including any lesion). If the pool contained tissues apparently free of disease, and a tuberculous lesion was visible elsewhere, then a portion of that lesion was added to the pool.

During the necropsy, a 2 g portion of both the left and right mammary glands were collected for culture if there was evidence of hypertrophy or milk secretion. Tracheobronchial lavage fluid was also gathered after clearing the trachea of surrounding muscles and making an incomplete transverse incision through the cranial portion. Lavage was performed using a tom cat urinary catheter attached to a syringe containing about 6 ml of sterile saline. The saline was forcibly ejected from the end of the catheter into the distal trachea and bronchi, saline was then aspirated from the airways and the process repeated, with the aim being to dislodge cellular debris and mucus before final aspiration and subsequent storage of the lavage liquid in a plain glass blood collection tube. If urine was present in the bladder, this was aspirated by needle puncture and stored in a plain blood collection tube. An attempt was made to massage faeces from the colon towards the rectum. If successful, a cut was made in the distal colon to allow collection of the faeces into a sterile pottle. If insufficient faecal material was present, then a swab was passed through the rectum, into the colon, thus ensuring that some material was available for culture.

The jaw was removed from 197 ferrets (87%), and the canine teeth sectioned and examined for the presence of incremental lines in the cementum, according to the technique described for polecats (*Mustela putorius putorius*) by Grue and Jensen

(1979). Sectioning data was used to aid accurate age determination, as it is difficult to differentiate the young of the year from adults, after they have reached 5-6 months of age.

Mycobacteriology

All tissues, swabs and fluids collected were removed using routines developed to ensure that the likelihood of cross-contamination between samples or between animals was minimised. These samples were held frozen in individual sterile containers at -84 °C until they were cultured for mycobacteria at the AgResearch Tuberculosis Laboratory, Wallaceville, using techniques described by Buddle *et al.*, (1994) and Jackson *et al.* (1995). All of the pooled tissue samples from each ferret were submitted for culture. The additional samples from individuals were only submitted for culture if *M. bovis* was isolated from the pooled lymphatic tissues.

Histopathological examination and data recording procedures

The entire opened carcasses of many of the ferrets, especially those which had not previously been frozen, were preserved by immersion in 10% formol-saline and held at 4 °C awaiting further examination. Fifty-three ferrets that were known on the basis of culture to be infected with *M. bovis* were subjected to histopathological examination. A full range of tissues from 18 ferrets and liver from a further 37 ferrets, from which *M. bovis* was not isolated, were also examined histologically. These uninfected ferrets were chosen from a list of accession numbers of culture-negative animals without knowledge of other information about the individual. Up to 45 tissues (Table 3-II) were examined histologically from each animal, including all major lymph nodes, as well as the oropharyngeal (palatine) tonsils, sections from each lung lobe, the liver, kidneys, spleen, adrenals, thymus, pancreas, duodenum, small intestine and colon. Testes or mammary glands from a few individuals were also examined. In the case of paired organs the two were distinguished. Tissue sections were processed routinely, sectioned at 5 µm and duplicate slides were stained with haematoxylin and eosin (H&E) and Ziehl-Neelsen (ZN) stains. Van Gieson stain for connective tissue was used on selected sections. Peripheral lymph node sections were also examined with the aid of a polarising microscope to assist identification of foreign material or debris in the tissues.

As a measure of the number of AFB in lesions, the number visible in each of ten 400 X magnification fields (high power fields, hereafter denoted HPF) chosen to predominantly contain macrophages was counted on slides stained with ZN. The mean number of AFB for each infected tissue was calculated. The resulting data allowed categorisation of the sites into those with means of; < 1 AFB, 1 - 10 AFB, 11 - 100 AFB, and those with >100 AFB per HPF. To quantify the occurrence of granulomas within the liver, the number observed in ten arbitrarily chosen 200 X magnification fields (low power fields, hereafter denoted LPF) on slides stained with H&E was counted and the mean calculated. In each of the above calculations, if granulomas or AFB were present within the section but none were found in the ten chosen fields, a value of 0.1 (i.e. one in ten fields) was assigned.

Liver 'biopsy' examination

A biopsy technique was investigated to determine whether the examination of small sections of the liver would furnish a sensitive test for tuberculosis in live animals. All 18 ferrets used were dead, laid in dorsal recumbency and had a 2mm incision made through the skin of the right abdomen immediately caudal to, and in the midsection of the costal arch. An 18 gauge biopsy needle (Biopty-Cut Needle, C. R. Bard Inc., Covington) attached to a biopsy gun was inserted through the skin incision, and pushed 10 mm cranial, whilst angled slightly toward the centre line, into the caudate and right lateral lobes of the liver. The sample was collected and treated in a similar manner to the other specimens for histopathology. Haematoxylin and eosin sections were examined, without prior knowledge of the infection status of the ferret, for the presence of granulomas. The findings were compared with the microbiological results and alternative forms of histopathological examination.

Statistical analysis

A variety of statistical procedures were applied to the data and, unless otherwise stated, the statistical software package SPSS version 7 (SPSS Inc., Chicago) was employed. Where appropriate, 95% confidence limits have been presented with results. All contingency table analyses were performed within the program Epi Info version 6.02 (Centers for Disease Control and Prevention, Atlanta).

Unconditional logistic regression was also used to investigate the association between the presence of pulmonary "lipid plaques" and disease status, whilst

controlling for sex and age. The initial model, as well as including these main effects as dependent variables, also contained the first order interaction terms of age, sex and disease status. Backwards stepwise elimination, based on maximum partial likelihood estimation, was used to remove non-significant terms from the model.

The relationship between the number of AFB and the presence of necrosis within lymph node lesions was examined by χ^2 analysis for trend. Counts of lesions containing AFB were made, and categorised according to whether they were simple granulomas, or granulomas with necrosis. These were further categorised according to the number of AFB observed per HPF.

Associations between lesions occurring in six body regions were investigated using log-linear analysis. Fifty-four cases for which detailed histology was available were used to establish relationships between the regions in this multi-way frequency analysis. A hierarchical modelling approach using backward elimination for parameter selection, starting from a model including all main effects and first order interaction terms, was employed. Removal of a term was based upon each subsequent subset model having the largest probability of significance. The end result was a parsimonious model, reduced to a small group of two-way interactions which provided acceptable goodness of fit to the data.

The designated regions used in the log-linear model were; HEAD, which included the oropharyngeal tonsils, mandibular and retropharyngeal lymph nodes; GUT, which included the jejunal, colonic and gastric lymph nodes, the duodenum, small intestine and colon; PERIPH, which included the superficial axillary, paired deep axillary, popliteal, inguinal, subiliac (prefemoral), and internal iliac lymph nodes; LUNG which included only lung lesions with AFB, and lesions of the mediastinal or bronchial lymph nodes; HAEM, which included the spleen, thymus, kidneys, adrenal glands and bone marrow; and PRESCAP, which included only the caudal cervical lymph nodes. The liver and the hepatic lymph node were not included in this analysis as the very high frequency of occurrence of lesions in this region would have precluded any useful outcome from this term's incorporation in the model. For analytical purposes, lesions were defined as microscopic granulomas or foci of necrosis attributable to tuberculosis (excluding lung granulomas without

AFB), or sites containing AFB independent of lesions. These lesions are hereafter denoted TbLs.

Investigating associations with the stage and severity of disease

For the purpose of analysis, the severity of disease was categorised into three stages i.e. advanced, where there were more than ten TbLs, or more than two gross lesions (excluding those of the lungs); “early”, where there were ten or fewer TbLs, or two or fewer gross lesions apparent; and negative, where no evidence of disease was found. Note that the classification of lesions as being “early” has been used in the manuscript for convenience and could be better described as “non-advanced”. The term should not be taken literally to imply some chronology to the disease process, as non-advanced lesions may have been present in one animal for a similar length of time to advanced lesions in another.

The relationship between the median number of liver granulomas found in the advanced and “early” stage disease groups was investigated in 54 ferrets using the Mann-Whitney U test procedure.

The relationship between stage of disease and the body weight of ferrets ($n = 215$) was investigated with the aid of analysis of covariance (ANCOVA). The stage of disease was divided into only two categories, i.e. advanced and other. The “other” category included “early” cases as well as animals apparently free of infection. \log_e body weight (g) was the dependent variable in the model. Covariates included; \log_e length (mm), estimated age (months) \log_{10} transformed; and categorical variables examined included season, sex, and stage. A full set of main effects and all first order interaction terms were initially screened, and the least significant terms removed from the model until only those significant at the 5% level remained.

A standardised measure of the “condition” (indicator of fat and protein reserves) of each ferret was required to allow this variable to be screened in the poisson regression analysis described below. As the sexes are dimorphic, two equations were developed whereby the relationship of weight to length could be established. The relationship can be expressed by the general form:

$$\log_e \text{ Weight} = a + b * \log_e \text{ Length}$$

where the value of b can be readily determined by linear regression. Condition indices (CIs) were derived for each animal after fitting the values for weight and length into the resulting equations. The CIs were standardised across the sexes by dividing the individual CIs by the mean for disease free individuals of that sex.

The poisson regression procedure of the statistical software package Statistix (version 4.1, Analytical Software, Tallahassee) was used to test associations between TbLs and other individual attributes which are likely to be recognised or measured at necropsy. The independent variables investigated included age, standardised CI, maturity, sex, presence of necrotic lesions, presence of pulmonary plaques and the number of gross lesions (not including lung lesions). Backwards elimination was employed to remove non-significant variables.

Analysis of covariance was used to test for an association between the mass of the spleen and infection with *M. bovis*. Spleen weight ($n = 138$), \log_e transformed, was used as the dependent variable. The body weight, estimated age (months), and body length (mm) were used as covariates, and season, sex, and stage of disease (negative, “early” and advanced disease) were the categorical independent variables investigated. All first order interaction terms were also screened, and the final model incorporated terms significant at the 5% level only.

Results

Of 228 ferrets examined, culture or histopathological results were available for 220. Seventy-three ferrets were classified as diseased, giving an overall prevalence of 0.32 (0.26 - 0.38) in the sample. *Mycobacterium avium* was isolated from one other animal with no gross lesions. In three ferrets, diagnosis was based on histological lesions consistent with tuberculosis, and in all other cases *M. bovis* was isolated. The sample was composed of 128 males (including 35 juveniles under 6 months of age) and 98 females (including 28 juveniles). One cannibalised animal was not categorised by sex, and one other juvenile male, diagnosed tuberculous by pharyngeal swab culture, was captured and released with a radio-collar, but not recovered for necropsy.

Necropsy

No gross lesions were seen in 27.8% (20/72) of tuberculous ferrets. Suspicious lesions were observed in 11.1% (8/72) of the diseased animals, and typical gross lesions were present in the remainder (61.1%) of the diseased ferrets. Two cases of multifocal lymphoid hyperplasia, and one of lymphoma were falsely categorised as tuberculous at necropsy through having multiple enlarged lymph nodes. Of the 54 animals which were subjected to extensive histopathological examination, the number of gross lesions varied between 0 and 15 (mean = 2.3; median = 1.0), 16 had no gross lesions (29.6%), 16 had only a single gross lesion, and 15 (27.8%) had two gross lesions.

Gross changes in lymph nodes of tuberculous ferrets examined were generally unspectacular compared with the pyogranulomatous or caseous lesions in other wild species such as possums and deer. Occasionally darkly pigmented nodes were visible in the caudal half of the animal, especially the jejunal and inguinal lymph nodes, but their presence was unrelated to disease state.

Table 3-II. Prevalence of gross lesions, distribution of acid-fast bacilli, granulomatous inflammation and necrosis within granulomas of lymph nodes (Lnn) and other organs of 54 ferrets with tuberculosis

Tissue	Gross lesions (%)	No. sites examined ^a	Acid-fast bacilli (%)	Granulomatous inflammation	Necrosis (%)
Jejunal lnn	46	54	32(59)	39(72)	28(52)
Retropharyngeal lnn	21	102	33(32)	40(39)	18(18)
Colonic lnn	2	53	17(32)	18(34)	9(17)
Caudal cervical lnn	17	106	28(26)	40(38)	23(22)
Liver	0	54	12(22)	50(93)	6(11)
Bronchial lnn	3	49	11(22)	21(43)	6(12)
Mandibular lnn	13	97	21(22)	23(24)	12(12)
Superficial axillary lnn	13	102	21(21)	33(32)	13(13)
Popliteal lnn	16	103	20(19)	28(27)	11(11)
Inguinal lnn	8	98	19(19)	27(28)	9(9)
Mediastinal lnn	0	48	9(19)	13(27)	6(13)
Hepatic lnn	0	48	8(17)	13(27)	4(8)
Lung ^b	76	54	9(17)	43(80)	0(0)
Gastric lnn	0	52	8(15)	8(15)	2(4)
Spleen	0	54	7(13)	15(28)	3(6)
Subiliac lnn	0	61	7(11)	10(16)	4(7)
Internal iliac lnn	3	98	11(11)	18(18)	6(6)
Deep axillary lnn	3	101	11(11)	21(21)	7(7)
Small intestine	0	54	4(7)	3(6)	0(0)
Bone marrow		53	3(6)	4(8)	0(0)
Adrenal gland	0	106	6(6)	4(4)	2(2)
Thymus	0	53	3(6)	1(2)	1(2)
Colon	0	54	3(6)	3(6)	0(0)
Oropharyngeal tonsil	0	103	4(4)	7(7)	0(0)
Duodenum	0	53	2(4)	1(2)	1(2)
Kidneys	3	108	4(4)	8(7)	3(3)
Testes	0	12	0(0)	0(0)	0(0)
Pancreas	0	52	0(0)	0(0)	0(0)
Mammary gland	0	19	0(0)	0(0)	0(0)

a Includes left and right sides where appropriate.

b Granulomatous inflammation not universally caused by tuberculosis.

Tuberculous lymph node lesions often included simple enlargement which was difficult to distinguish from normal hypertrophy. Focal white nodular lesions were occasionally seen in the node parenchyma but these tuberculous lesions were grossly indistinguishable from follicular hyperplasia. Some affected nodes were oedematous. In many diseased nodes, circular cream coloured foci were apparent. These varied from pin head size, up to larger lesions many millimetres in diameter which often coalesced (Figure 3-1). These focal lesions were often multiple, and found typically in subcapsular locations making them visible to the careful observer before node transection (Figure 3-2). The larger lesions, especially of the jejunal lymph node, often became liquefactive, turning the node into a watery or milky fluid filled sac. The jejunal lymph node sometimes reached a diameter of 35 mm when distended by liquefied contents, some of which occasionally leaked into the abdominal cavity producing a tuberculous peritonitis, characterised by a thin grey film of adherent exudate on the serosal surfaces. In one case each a tuberculous popliteal and an inguinal lymph node had fistulae draining through the skin.

Subpleural cream-coloured plaques up to 6 mm in diameter were found in the lungs of 51% of ferrets (Figure 3-3). These discoid lesions were typically seen on the dorsal diaphragmatic lobes, but when numerous were often spread evenly over all lobes. Large lesions were up to 1 mm thick and palpable, and developed a darker olive-grey centre with a rim of paler tissue. These plaques did not extend into the parenchyma, but were often associated with the presence of smaller focal cream-coloured lesions within the lung. Plaques were not visible in any animal under 4 months.

Plaques were 2.4 times as likely to be found in infected as uninfected ferrets ($p = 0.01$), with the prevalence increasing significantly with age (Table 3-III), such that for each 6 month age increment there was 2.5 times the risk of displaying plaques. Their occurrence was not significantly related to the area of origin, sex, nor any of the interaction terms investigated. Of those ferrets with plaques, lesions over 2 mm in diameter were more likely to be found in ferrets with tuberculosis ($\chi^2_1 = 10.0, p = 0.002$), the relative risk being 2.1 (1.3 - 3.5).

Table 3-III. Final logistic regression model examining the effect of age (months) and disease status on the presence of “lipid plaques” (n = 216)

Variable	Coefficient	SE	p-value	Odds ratio (95% CI)
Intercept	-1.64	0.32		
Age	0.15	0.03	<0.001	1.16 (1.09-1.24)
Infected	0.89	0.35	0.01	2.44 (1.24-4.81)

Deviance = 250.7, $\chi^2_2 = 48.6$, $p < 0.001$.

Lesions, associated with infection by adiaspores of the fungus *Chrysosporium parvum* var. *crescens* (previously known as *Emmonsia parvum* var. *crescens*), usually appearing as 1-2 mm palpable spherical glassy lesions of the pulmonary parenchyma and pleura, were commonly noted at necropsy (Figure 3-4). These granulomas were diagnosed in 19 of 71 (26.8%) ferrets examined histologically. In some animals, these lesions were present concurrently with plaques, and on occasion were so numerous that they resembled miliary pulmonary tuberculosis, as seen in other species, and have the potential to produce false diagnoses.

Poisson regression analysis examining the effect of factors seen or measured at necropsy in contributing to the number of TbLs, was performed after determining the standardised CI. The coefficient ‘b’ calculated for the length:weight ratio was, for males and females respectively, 2.686 and 2.857. Table 3-IV shows the results of the most parsimonious model incorporating all significant main effects. Approximately half of the deviance in TbL is explained by the model, which shows that there is a positive predictive value for the presence of plaques, number of gross lesions and presence of necrotic lesions on the number of microscopic lesions. The standardised condition index was negatively correlated with the number of lesions. Age, maturity and sex had no significant effect on the severity of disease.

Table 3-IV. Reduced poisson regression model showing main effects which significantly influenced the total number of lesions (TbLs)

Independent variable	Coefficient	SE	p - value
Constant	1.90	0.35	<0.001
Lipid plaques	0.41	0.14	0.004
Standardised CI ^a	-1.23	0.27	<0.001
Gross lesion no.	0.08	0.01	<0.001
Necrosis	0.97	0.21	<0.001

Deviance 128.9, $p < 0.001$, $df = 49$: a Condition index.

Table 3-V presents the analysis of deviance of the final model, used to assess whether the deletion of selected components will have a deleterious effect on the goodness of fit. Each of the four components tested caused a significant reduction in the fit of the model indicating that each of the terms was required in the model.

Table 3-V. Analysis of deviance testing the validity of inclusion of each term in the reduced poisson regression model (above)

Model ^a	Deviance	Difference ^b	DF	Components tested	p - value
Intercept(I)	296.6	167.7	4	P,CI,G,N	<0.001
I,CI,G,N	137.7	8.8	1	P	<0.001
I,P,G,N	149.4	20.5	1	CI	<0.001
I,P,CI,N	175.0	46.1	1	G	<0.001
I,P,CI,G	156.3	27.3	1	N	<0.001
I,P,CI,G,N	128.9				

a abbreviations for terms of the model are: I = intercept, CI = standardized condition index, P = lipid plaques, G = gross lesions, N = necrotic lesions.

b difference in the deviance between the full model and the same model with the indicated component removed.

The investigation of factors affecting body weight concluded with the model presented in Table 3-VI, which incorporated the explanatory variables, stage of disease, \log_e length, \log_{10} age, season and sex, and the interaction terms of sex*stage, sex*season, stage*length and stage*season. This model explained about 91% of the variation in weight and, of the predictor variables, length explained over half the variance. Ferrets in an advanced stage of the disease had a mean (least square) body weight of 785 ± 27 g ($n = 24$) whereas the remainder had a mean

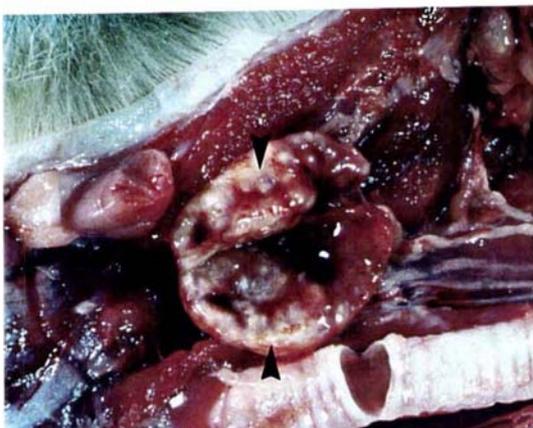


Figure 3-1. Transected tuberculous retropharyngeal lymph node showing oedema, and haemorrhagic congestion subsequent to a lethal blow on the head, and extensive peripheral necrotic granulomatous foci (arrowheads).



Figure 3-2. Infected jejunal lymph node with slight enlargement, showing characteristic pale subcapsular foci (arrowheads) associated with necrosis of granulomas.



Figure 3-3. Large pale subpleural “lipid plaques” on the caudo-dorsal lung surface. Note the darker centre of the largest plaque (arrowhead).

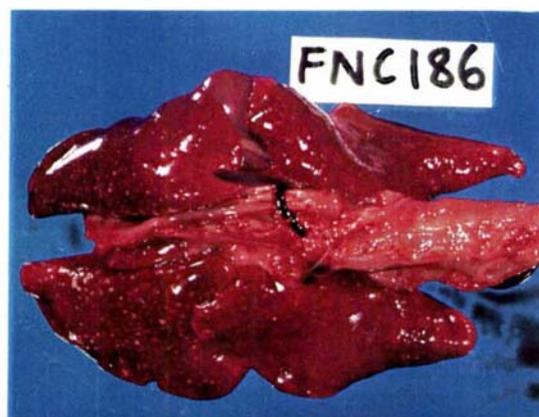


Figure 3-4. Lung showing extensive small focal adiaspiromycotic granulomas caused by *Chrysosporium* spp.

weight of 885 ± 11 g ($n = 191$). The sex of the ferret also had a significant effect on the amount of weight loss in advanced cases, as males with advanced disease showed a greater differential in weight (206 g) than females (5 g) with advanced disease, when compared with the balance of the population of the same sex. Female weight, when adjusted for the other factors in the model, seemed to remain steady throughout the four seasons, whereas the male weight, although consistently higher than the female, dropped by about 100 g in the winter and summer. The “stage” interaction terms in the model need cautious interpretation, as the data were unbalanced, and the standard errors of the means were large.

Table 3-VI. Model of significant factors which influenced \log_e body weight in 215 ferrets

Parameter	DF	F - value	p - value	Eta squared ^a
Season	3	0.70	0.551	0.01
Sex	1	1.6	0.203	0.01
\log_e length	1	142.8	<0.001	0.42
\log_{10} age	1	4.7	0.031	0.02
Stage	1	10.5	0.001	0.05
Stage*season	3	6.8	<0.001	0.09
Sex*season	3	3.1	0.028	0.04
Stage*sex	1	7.0	0.009	0.03
Stage*length	1	10.3	0.002	0.05

Adjusted $R^2 = 0.91$: a Estimate of the variance explained by term in model.

Splenomegaly was noted in a number of ferrets. Investigation of this phenomenon resulted in the model presented in Table 3-VII. Significant explanatory variables included, season, weight and stage of disease, and the interaction term season*weight. This model had an adjusted R^2 of 0.68 indicating that these factors explained about two thirds of the variance in spleen weight. Examination of the least squares means showed that spleen weights were heaviest in summer and winter, and lightest in spring and autumn. A helmert contrast applied to the three stages of disease, showed that the weight difference due to stage was significant only between advanced disease ($n = 17$) and the other two categories combined ($n = 121$). The amount of variance explained by disease stage was small (6%), in comparison with body weight which explained over half the variance. The least squares means (back-transformed) for the three disease stage categories, advanced,

“early” and negative were, respectively 8.3 g, 7.0 g and 6.7 g. The median and range for spleen weight in the advanced disease stage group were 10 g, 3-19 g. For the remainder of the ferrets the equivalent values were 6 g and 2-18 g. Of the ferrets with the ten highest spleen weights, there were three with advanced disease, five with “early” disease and two without infection.

Table 3-VII. Results of analysis of covariance of the spleen weight (\log_e transformed) of 138 ferrets

Model variables	DF	F - value	p - value	Eta squared
Weight	1	169.8	<0.001	0.57
Season	3	12.0	<0.001	0.22
Stage of disease	1	4.2	0.018	0.06
Season*weight	3	12.4	<0.001	0.23

Histopathological findings

Microscopic lesions were found in all of the animals from which *M. bovis* was isolated (hereafter called infected) and AFB were identified in at least one tissue from 48 of the 54 ferrets known to be infected (Table 3-II). The number of sites in which AFB were found in infected ferrets ranged from 0 to 31 (median = 2.50; mean = 5.76). Acid-fast bacilli were found in a single site in ten animals. The number of TbLs ranged from 1 to 32 (median = 6.0; mean = 8.7). Two infected animals found dead, an animal that was wandering abroad in daylight and one other killed by a vehicle had TbLs involving 32, 28, 24 and 24 sites, respectively. No lesions, other than those associated with tuberculosis, were identified to account for the death or possible illness of these animals.

Lymph nodes

A common microscopic change in the lymph nodes of both infected and uninfected ferrets was the presence of increased numbers of macrophages within the sinuses. These cells had pale eosinophilic cytoplasm and were arranged along the medullary sinuses. This was regarded as a non-specific inflammation described as "sinus histiocytosis" or "histiocyte hyperplasia" by Jubb *et al.* (1993). Very small numbers of AFB were occasionally found in these areas in some infected animals but, because this change was also common in lymph nodes of some uninfected animals, it will not be considered further here as a tuberculous lesion. Discrete aggregations

of larger macrophages with extensive eosinophilic cytoplasm and large vesicular nuclei (epithelioid cells) were present in one or more lymph nodes in 51 of the diseased animals. These aggregates varied from a few cells (Figure 3-5) to extensive sheets of cells that occupied much of a lymph node (Figure 3-6). For purposes of quantification, these were defined as sites of granulomatous inflammation (Table 3-II). Aggregations of epithelioid cells were not found in lymph nodes or other tissues of any animal that was negative by culture. Many epithelioid cell aggregations surrounded a central core of amorphous necrotic tissue and were surrounded in turn by a thin margin of lymphocytes with lesser numbers of plasma cells. The distribution of foci of necrosis is shown in Table 3-II. In the animals examined AFB were rarely detected within the necrotic debris in most such foci but were present in epithelioid cells at the margin of the necrotic areas. In most animals examined, and in most individual lesions, AFB were present in small numbers and individual macrophages rarely contained more than one AFB.

There was a strong and significant trend ($\chi^2_3 = 96.1$, $p < 0.001$) for lesions with necrosis to contain higher numbers of AFB (Table 3-VIII). Lesions containing > 100 AFB/HPF were found in 20 ferrets. The number of sites in which AFB were found in these animals ranged from 1 to 31. The two animals that had been found dead, the animal that was acting abnormally and the road casualty had 55% of all lesions that contained > 100 AFB/HPF.

Fibrosis was not prominent in any of the lymph node lesions, except in a few instances where there was focal fibrosis of the capsule adjacent to a necrotic area. Neutrophils were infrequent within areas of granulomatous inflammation or necrosis in most animals. One ferret had an extensive pyogranulomatous focus with a central core of necrotic material in a superficial axillary lymph node; AFB were numerous in this lesion. Mineralisation or giant cells were not detected in lesions containing AFB in any of the infected animals.

Table 3-VIII. Chi² analysis for trend, examining the association between presence of necrosis in lymph node lesions, and the abundance of AFB, in 54 infected ferrets

Number of AFB ^a	Granulomas with necrosis	Granulomas without necrosis	Odds ratio
< 1	11	48	1.00
1 - 10	15	18	3.64
11 - 100	25	16	6.82
> 100	101	7	62.96

a Average number of AFB observed per 10 high power microscope fields.

χ^2_3 for linear trend = 96.1, $p < 0.001$.

Liver

Microscopic lesions were detected more commonly in the liver of the infected ferrets than in any other single site (Table 3-II), although macroscopic hepatic lesions were not detected. The predominant change consisted of nodular accumulations of macrophages randomly distributed throughout the parenchyma (Figure 3-7). The number and size of these micro-granulomas varied considerably and some contained small numbers of lymphocytes, plasma cells and occasionally neutrophils, in addition to macrophages. The average number of nodules per LPF in individual infected animals ranged from 0 to 27.6. Acid-fast bacilli were detected within these granulomatous foci in 12 animals.

The median number of liver lesions (3.5, range 0.4-27.6) in the advanced disease group was significantly greater (Mann-Whitney U = 496.5, $p < 0.001$) than the median (0.65, range 0-21.4) of the "early" group (Mann-Whitney U = 111.5). In individual animals, the greatest number of hepatic granulomas was typically associated with advanced necrotic lesions containing many AFB, in the jejunal, colonic and gastric lymph nodes. Two of the four cases in which liver lesions were not detected had one or two isolated necrotic lesions in peripheral body nodes only. The other two cases had granulomas without AFB in either a mandibular or jejunal lymph node only. None of these four cases had gross lesions at any site (including lungs).

The livers of 55 infected, and 51 uninfected ferrets were examined for the presence of granulomas. Fifty-two livers contained lesions, and 51 of these were from infected animals. Four livers from infected animals were without granulomas. If

the presence of liver granulomas were used as a diagnostic test, then the apparent sensitivity when compared to the culture of pooled lymphatic tissue, was 92.7% (81.6-97.6), and the apparent specificity 98.0% (88.2-99.9).

Liver samples were collected from 18 ferrets using a "biopsy" method on the carcass. Definitive and suspicious lesions, including granulomas, or small aggregations of lymphoid, epithelioid or necrotic cells, were found in five of seven infected animals, and in two of eleven uninfected animals. If these lesions were considered as positive disease diagnoses, then the apparent sensitivity and specificity of this diagnostic technique were, respectively, 71.4% (30.3-94.9) and 81.8 (47.8-96.8).

Lung lesions

The prevalence of macroscopic and microscopic pulmonary lesions related to *M. bovis* was difficult to interpret because of the frequent occurrence of granulomatous inflammation in both infected and uninfected ferrets. The most common change was consistent with lesions described as "endogenous lipid pneumonia", "foam cell pneumonia", or in more advanced cases, as "cholesterol pneumonia", by Jubb *et al.* (1993), but which are now known as foci of "alveolar histiocytosis". These lesions, appearing grossly as subpleural plaques, ranged from circumscribed aggregations of foamy macrophages within alveoli, usually in a subpleural location, to large more discrete aggregations containing both foamy and granular macrophages with limited fibrosis and accumulation of lymphocytes, plasma cells and occasional eosinophils (Figure 3-8). Many of the larger lesions in the parenchyma contained cholesterol crystals (Figure 3-9). Another lesion type contained intralesional structures consistent with adiaspores of the fungus *Chrysosporium parvum* var. *crecens*. Spores were often surrounded by a mixed cell population of lymphocytes, plasma cells and macrophages with occasional giant cells (Figure 3-10). Lesions containing adiaspores were found in the lungs of twelve infected and seven uninfected ferrets. Five animals had lesions containing an adiaspore in a bronchial or mediastinal lymph node, and in three of these spores were not found in lung sections. One infected ferret had inflammation associated with an unidentified nematode in the pulmonary parenchyma. Lesions containing AFB were found in the lungs of nine ferrets and ranged from small discrete collections of both foamy macrophages and epithelioid cells within alveoli to the presence of a few epithelioid cells containing

AFB at the periphery of large lesions typical of “advanced alveolar histiocytosis”. Necrosis was not associated with the pulmonary lesions containing AFB. Of the ferrets in which AFB were found in the lungs, seven had granulomas containing AFB in bronchial or mediastinal lymph nodes; one had granulomatous inflammation in the bronchial nodes but AFB were not found, and the remaining animal had no granulomatous reaction visible in the bronchial nodes. Eight additional animals had AFB within their bronchial or mediastinal lymph nodes without AFB being found in the lungs.

Other sites

Lesions in spleen, tonsils, adrenals, and bone marrow consisted of small discrete aggregations of epithelioid cells with very few AFB, except in one animal found dead in which there were areas of necrosis containing many AFB in spleen and bone marrow. The tonsillar lesions did not appear to involve the lymphoepithelium, and lay adjacent to the follicles (Figure 3-11).

Five ferrets had renal lesions. In four cases the lesions were bilateral, with three of these having collections of lymphocytes and plasma cells, with occasional macrophages, in the interstitium of the cortex, and one only having AFB visible. The other ferret with bilateral lesions had areas of cortical necrosis with numerous AFB. The ferret with the unilateral lesion had a necrotic focus containing numerous AFB. Four of the ferrets with kidney lesions were classified as having advanced disease. Interstitial nephritis and small renal cysts, up to 1 cm in diameter, were occasionally noted, but their presence was unrelated to disease status.

Because of the method of preservation, sections of the intestine contained considerable artefact. Lesions containing AFB were found in two animals, and consisted of infiltration of the lamina propria and submucosa with a mixture of lymphocytes and macrophages (Figure 3-12). No lesions were found in pancreas, mammary gland or testes.

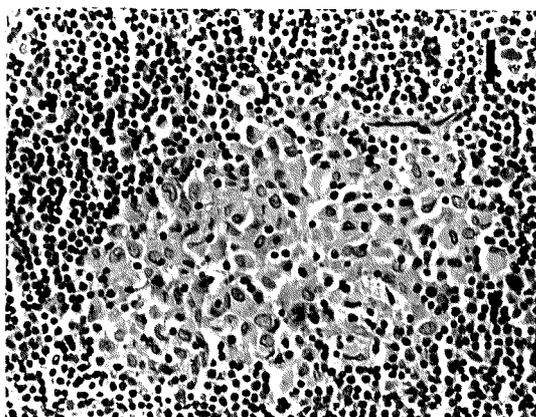


Figure 3-5. Small granuloma without evidence of necrosis, in the internal iliac lymph node. H&E. Bar = 25 μm



Figure 3-6. Relatively small granulomas within a mandibular lymph node of a ferret with widely disseminated tuberculosis. A small focus of necrosis is visible in the centre of one granuloma (arrowhead). H&E. Bar = 100 μm .

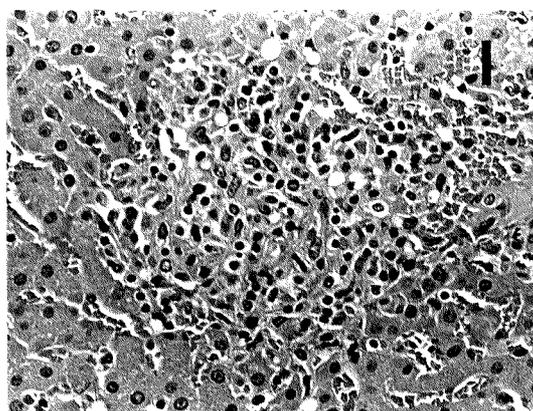


Figure 3-7. Focal granuloma within the liver of a ferret with tuberculous lesions in several lymph nodes. The reaction consists predominantly of macrophages with a few lymphocytes. H&E. Bar = 25 μm .

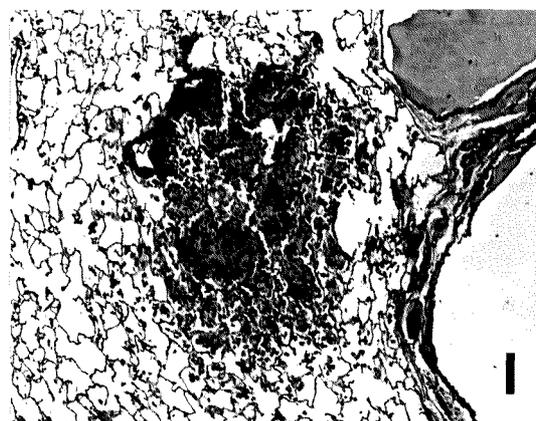


Figure 3-8. Small discrete pulmonary granuloma consisting predominantly of foamy lipid-laden macrophages, small numbers of AFB are present. Linear gaps in the granuloma are artefactual. H&E. Bar = 100 μm .

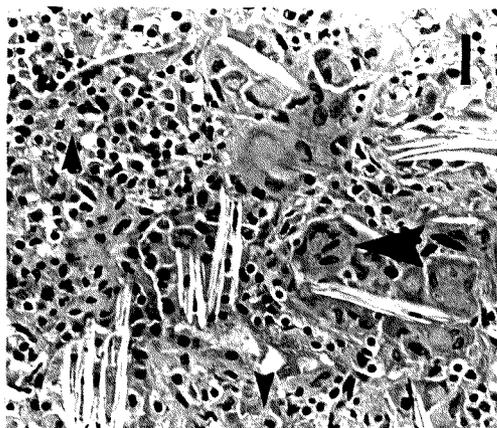


Figure 3-9. Granuloma within the lung comprised of foamy macrophages (small arrowheads), macrophages with more dense cytoplasm, occasional multinucleate giant cells (large arrowheads), lymphocytes and plasma cells. Numerous cholesterol clefts are present. H&E. Bar = 25 μm .

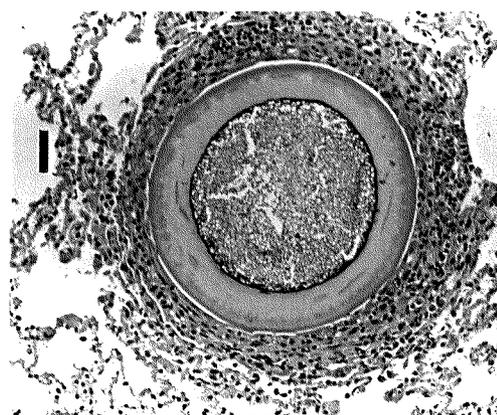


Figure 3-10. Pulmonary granuloma containing a large thick walled spore of *Chrysosporium* spp. The macrophages surrounding the spore have a uniform pale eosinophilic cytoplasm. H&E. Bar = 50 μm .

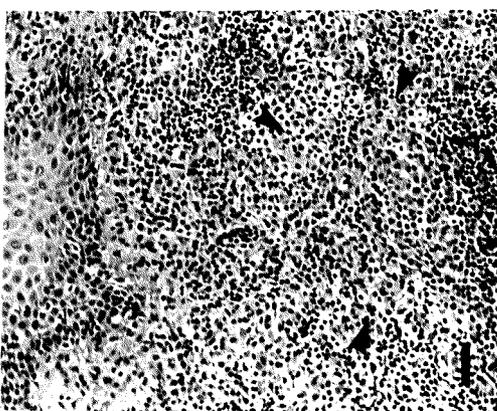


Figure 3-11. Granuloma (arrowheads) overlying lymphoid follicle in the oropharyngeal tonsil of a ferret with disseminated tuberculosis. Small numbers of AFB were present. H&E. Bar = 50 μm .



Figure 3-12. Discrete aggregations of lymphocytes (small arrowheads), and macrophages (large arrowhead), in the submucosa of the duodenum of a ferret with disseminated tuberculosis. A few AFB were present within macrophages. H&E. Bar = 50 μm .

Regional relationships

The best fitting log-linear model of associations between lesion sites included the five interaction terms: HEAD x LUNG; HEAD x GUT; HEAD x PRESCAP; LUNG x PERIPH; and LUNG x HAEM. This indicates that there is a significant association between the presence of lesions at each of the two sites included in each interaction term. The significance levels for goodness of fit for this model were 0.52 (likelihood ratio $\chi^2_{52} = 50.8$) and 0.57 (Pearson $\chi^2_{52} = 49.5$). Both the likelihood ratio and the Pearson χ^2 statistics show acceptable goodness of fit for the model selected, and a scatterplot of the observed versus expected frequencies showed no major outliers in the data.

To establish the magnitude of these interactions, it was necessary to perform 2 x 2 contingency table analysis to derive the associated relative risks. The calculated values are presented in Table 3-IX, and show that animals which have lesions in the head, are twice as likely to have lesions in the lung or associated lymph nodes, 1.5 times as likely to have lesions in the gut or associated lymph nodes, and 2.2 times as likely to have lesions in the caudal cervical lymph node, as those that do not. Animals which have lesions in the lung, are twice as likely to have lesions in the superficial nodal sites, and 2.3 times as likely to have identifiable lesions in the spleen, kidney, adrenal glands or bone marrow, as those that do not.

Table 3-IX. Relative risks for significant associations between lesion sites included in the log-linear model

Interacting lesion sites	Relative risk (95% CI)	p-value
HEAD x LUNG	1.96 (0.95-4.05)	0.041
HEAD x GUT	1.50 (0.98-2.29)	0.030
HEAD x PRESCAP	2.16 (1.06-4.40)	0.014
LUNG x PERIPH	1.96 (0.95-4.05)	0.041
LUNG x HAEM	2.33 (1.38-3.94)	0.002

Discussion

Gross lesions were most prevalent in the jejunal lymph node, being found in nearly half of the infected animals. No other site of gross lesions approaches the same frequency of occurrence, the nearest being the retropharyngeal lymph node with a prevalence of 0.21. This high frequency of jejunal lesions has been found in previous reports of the disease in ferrets in New Zealand (Ragg *et al.*, 1995c), and provides strong evidence for the bulk of the infections having an alimentary tract origin.

Nearly one third of the infected ferrets had no gross lesions. This satisfactorily quantifies the poor sensitivity of identification of infected animals solely by necropsy procedures. Many of the “early” lesions seen were difficult to distinguish from the variable appearance of the normal lymph node and necrotic lesions were often so small as to be barely detectable by eye.

The higher prevalence of disease found in the current series of ferrets is at odds with earlier New Zealand studies (Ragg *et al.*, 1995b; Walker *et al.*, 1993; Cowan, 1994; Oliver, 1996), with the lower confidence limit in this study (0.25), being equal to the upper confidence limit of the highest previously reported. This circumstance has almost certainly arisen from inapparent infections being overlooked at necropsy, as all previous diagnoses were based on gross lesion identification at necropsy, with subsequent confirmation by histopathological or mycobacteriological examination. Some small and less advanced lesions will have been overlooked, and consequently many more AFB found in the advanced lesions that were examined. This, and earlier reports in the literature, has led to the belief that ferret lesions are characterised by enormous numbers of AFB (Symmers *et al.*, 1953; de Lisle *et al.*, 1993). A strong relationship between the abundance of AFB and the presence of necrosis has been demonstrated in this study, which also lends weight to the suggestion that “early” lesions with few AFB have been overlooked in the past.

The negative results of the poisson regression analysis are probably of most importance to understanding the disease process. The severity of disease was not related to age nor sex, indicating that disease progression is independent of these host factors. The presence of “lipid plaques”, especially large ones, has a role in alerting the examiner to an increased possibility of infection, but splenic enlargement is clearly not specific enough in its aetiology to be diagnostically

useful at necropsy. Low body condition may be associated with advanced disease, but in these cases there will be sufficient easily recognised lesions to guide the diagnosis. Despite intensive investigation there appears to be no substitute for the careful examination of lymph nodes for the presence of gross lesions, especially necrotic foci, to demonstrate the presence of infection at necropsy. Attention will be most usefully directed at the nodes with the highest prevalence of lesions in “early” cases (Table 3-II), and should include the retropharyngeal, mandibular, superficial axillary, caudal cervical, popliteal, inguinal, jejunal and colonic lymph nodes.

The data arising from the ANCOVA of body weight (Table 3-VI) suggested that male ferrets are prone to weight loss, especially in the winter when food is scarce and they are involved in mating and territorial activities, but also in the summer. Males also showed significant weight loss when affected by advanced disease, whereas the females were not significantly affected. It would seem that males are more prone to weight fluctuation from whatever cause, than are females. These findings must be interpreted cautiously, as only 24 ferrets with advanced disease existed in the data set, and because there is little available information on the duration of the disease process from infection to death. It seems likely that being tuberculous does not affect the general health of the ferret until the latter stages of disease. The ferrets with poorest condition in the study of Walker *et al.* (1993) had a higher prevalence of tuberculosis, which supports the notion of advanced disease affecting the health of many animals. Experimental infections have resulted in the ferrets appearing normal until a matter of days before death (Symmers, *et al.*, 1953), indicating a fulmination of the disease process in the terminal stages. Our data contained one dead and one sick tuberculous ferret which were in poor condition at death or shortly prior, suggesting that the terminal stage disease may progress slowly enough for body reserve depletion in at least some ferrets. One other ferret which appeared to have died from tuberculosis still had fat reserves remaining. In possums, body condition is maintained until the terminal stage of the disease (Jackson *et al.*, 1995a).

The splenomegaly appears to be an inconsistent finding in advanced disease states, and seems to be due to an increase in mass of the normal cellular elements, and the development of small granulomas in some cases. On average the magnitude of this

enlargement is only slight, and the effect is likely to be obscured by other unidentified causes of hypertrophy. A large spleen found during necropsy may be associated with advanced disease, but more specific lesions will also be evident. Typical splenic lesions have been simply described as aggregations of macrophages, up to 2.5 mm in diameter, appearing as ill-defined nodules (Symmers *et al.*, 1953; Thorns *et al.*, 1982). This is consistent with our observations. Symmers *et al.* (1953) did note an enormously enlarged spleen in one experimentally infected ferret. Splenomegaly is a consistent feature of the disease in mink, in which the disease process generally seems to be more severe (Pulling, 1952; Head, 1959; Adamesteanu *et al.*, 1970; Beck *et al.*, 1974).

No gross hepatic lesions were evident in the current series of ferrets, but these have been reported in one of 98 infected ferrets by Ragg *et al.* (1995c), and by Dunkin *et al.* (1929) in two naturally infected animals. Despite the lack of gross lesions, microscopic liver granulomas were the most prevalent lesion of infected ferrets and have been reported in all other cases where the liver has been examined. These granulomas may, however, be absent in early infections or in cases where the infection has been localised to a peripheral lymph node. Because of the very high specificity of these lesions we believe that they are pathognomonic for *M. bovis* infection, even in the absence of visible AFB. The high prevalence of these granulomas will make rapid diagnosis by histology a possibility for cross-sectional studies. The diagnostic potential of liver biopsy may have some experimental utility, and could be used as a tool in longitudinal studies. Sensitivity could be improved by the use of a wider bore biopsy needle, allowing the collection of a larger volume of liver. As all these granulomas will undoubtedly contain some AFB, sections of liver may be usefully incorporated into pooled tissue samples for mycobacteriology, or polymerase chain reaction (PCR) testing if a rapid definitive diagnosis is required.

None of the ferrets we examined had extensive nor advanced pulmonary lesions. The common occurrence of other forms of granulomatous lung disease means that histological examination of lungs for tuberculous lesions will not be diagnostic. Although one case of a caseating pulmonary lesion has been reported (Dunkin *et al.*, 1929), necrosis of pulmonary granulomas, even those containing AFB, was not present in the current series. The absence of necrosis and extensive lesions

indicates that the cell-mediated immunity of the pulmonary compartment, unlike many species, can provide an environment which is hostile to the survival of *M. bovis*, and also that primary infection of the lungs is rare. Ragg *et al.* (1995c) also found a low prevalence of pulmonary lesions (2.9%) attributable to tuberculosis. Symmers *et al.* (1953) believed that the entrapment of AFB in "lipid plaques", which are common in the genus *Mustela*, was secondary to their formation from unknown causes. We believe this to be true, but our findings also suggest that the presence of *M. bovis* in the pulmonary circulation may exacerbate pre-existing lesions and make visible those that would have hitherto been inapparent.

The results of the log-linear analysis allowed further strengthening of hypotheses regarding the pathogenesis of infection. The association between lesions in the head area and the caudal cervical lymph node is readily explained by the efferent lymphatic branch of the retropharyngeal node which passes to the caudal cervical (Shibata, *et al.*, 1988). Head lesions were also statistically associated with gut-associated lesions, which is consistent with the alimentary tract, including oral mucosal injuries, oropharyngeal tonsils and intestinal Peyer's patches, being the principal route of entry of bacilli when ingested. The association of head lesions with thoracic lesions is probably similar to the relationship between the peripheral nodes and the thorax, in that bacilli entering the circulation from these sites must first pass through the pulmonary capillary bed where they are likely to be engulfed by intravascular or intra-lesional macrophages. The association between the HAEM group of sites and thoracic lesions provides strong evidence that when spleen, bone marrow, kidney, and adrenal gland become involved, which can only be through haematogenous spread, the lung is also likely to be infected simultaneously. The almost universal infection of the liver, no matter what other sites were infected, demonstrates that bacilli readily escape from nodal lesions, and are distributed by the blood stream early in the course of infection and are trapped by the Kupfer cells lining the liver sinusoids.

It appears that the disease process for *M. bovis* infection in the ferret is not as aggressive and tissue destructive as it is in many other species in which the lesions are normally caseous in nature. The histological appearance of advanced lesions in the genus *Mustela* is characterised by macrophage accumulation, large numbers of AFB, especially at the periphery of necrotic centres, with many macrophages

containing multiple bacilli. Giant cells are not a feature, and caseation and fibrosis rare (Dunkin *et al.* 1929; Head, 1959; Adamesteanu *et al.*, 1970; Thoms *et al.*, 1982). Large numbers of *M. bovis* organisms have been found to be toxic for ferret lymphocytes *in vitro* (Thoms *et al.*, 1982), and also apparently for macrophages *in vivo*. The development of the cell-mediated response appears to be slow, and is possibly compromised by the presence of *M. bovis* antigens, which are capable of suppressing lymphocyte reactivity (Thoms *et al.*, 1982). Ferrets, once infected, keep the disease under control for an extended period of time, during which the lesions progress slowly, until sufficient *M. bovis* and their secreted antigens are present to cause a very severe depression of the cell-mediated immune response. The organism then replicates freely and death ensues, presumably from toxæmia.

Ferrets, because of their high prevalence of infection and widespread distribution, are potentially a very good indicator of wildlife infection in an area. As the capture yield is low it is worthwhile ensuring that those which are caught will be accurately classified with regard to tuberculosis status. Because many tuberculous ferrets will have inapparent infection at necropsy, it is essential that additional laboratory procedures are carried out on animals without lesions. Examination of the liver of all ferrets by histopathology is strongly recommended as a rapid and sensitive diagnostic test. Where tissues are being sent for culture in the absence of gross lesions, a pool of retropharyngeal, caudal cervical, jejunal lymph nodes and a small portion of liver should be collected for mycobacteriological examination, to ensure the greatest likelihood of including sufficient bacilli for successful isolation from a single pooled sample.

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The PhD candidate was responsible for the study design, execution of the vast majority of the necropsies and collection of samples for bacteriology, analysis of data and preparation of the manuscript. Gary Wobeser (on leave from the Canadian Co-operative Wildlife Health Centre, Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada.) performed the histopathological examinations, and Peter Caley (Landcare Research) provided a large number of ferrets collected from his North Canterbury study site.

CHAPTER 4

EPIDEMIOLOGY OF *MYCOBACTERIUM BOVIS* INFECTION IN FERAL FERRETS (*MUSTELA* *FURO*) IN NEW ZEALAND: II. ROUTES OF INFECTION AND EXCRETION¹

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Abstract

Detailed necropsies of 228 ferrets captured from eight areas in the North and South Islands provided material for an investigation into the epidemiology of tuberculosis in wild ferrets. Seventy-three of the 228 (32%) animals examined were diagnosed as tuberculous, by culture of pooled lymph nodes and detailed histopathological examination. The prevalence of bovine tuberculosis was 96% in 24 ferrets taken from areas in which tuberculous possums were common. None of 35 animals under 4 months of age were found to be infected, and the prevalence of infection was shown to rise with age, such that for each 6 month age increment there was a 2.8 times greater risk of becoming infected. The most common route of infection appeared to be via the alimentary tract, as 79% of 38 animals, in which the initial lesions could be reasonably determined, had these lesions associated with the digestive tract. Samples from potential sites of excretion from infected ferrets were submitted for culturing. The most common route of excretion was via the oral cavity, with *M. bovis* recovered from 15 of 64 (23%) oral swabs. *Mycobacterium bovis* was also isolated from four of 64 (6%) tracheobronchial lavage samples, ten of 63 (16%) faecal samples, two of 29 (7%) urine samples and one of 8 (12.5%) mammary glands.

The disease in ferrets appears to be principally maintained by ingestion of tuberculous carrion. Although a moderate number of ferrets excrete *M. bovis* orally, there appears to be only minor intraspecific transmission by bite wounding. The findings provided no evidence to support the occurrence of pseudo-vertical transmission.

Introduction

Mycobacterium bovis infection was first reported in wild ferrets (*Mustela furo*) in New Zealand from animals captured on the West Coast in the early 1970s (Stockdale, 1975), but without cultural confirmation. The disease was subsequently confirmed by the isolation of *M. bovis* from a ferret in the Taumarunui area in 1982 (de Lisle *et al.*, 1993). These early reports generated little interest in the species as a host of bovine tuberculosis, as it was believed that ferrets were simply spillover hosts (Morris and Pfeiffer, 1995), present in only low density in areas where the disease was endemic in brushtail possums (*Trichosurus vulpecula*). In such areas infection was also known to be present in substantial numbers of other free-ranging mammals, including deer (*Cervus* spp.) and wild pigs (*Sus scrofa*).

The suggestion in recent years that ferrets were responsible for transmission of *M. bovis* to farmed cattle and deer in some instances, resulted in a dramatic escalation in the number of ferret examinations, and tissue samples submitted for mycobacterial culture after 1992. This belief was given initial impetus by the Mackenzie Basin wildlife studies from 1990 to 1992 (Walker, *et al.*, 1993) which reported an association between the presence of infected ferrets and the occurrence of reactor cattle or deer, on properties where there were considered to be few or no possums.

Other North and South Island areas were subsequently identified in which ferrets were suggested as a possible cause of tuberculosis in domestic stock. These included areas where possum control operations were apparently failing to achieve an effective reduction of the reactor incidence rate (Cowan, 1994; Atkinson and Cowan, 1994; Ragg and Walker, 1996).

Currently available evidence has demonstrated a broad-scale relationship between the presence of tuberculosis in ferrets and livestock, in that infected ferrets have not been detected in areas without evidence of infection in susceptible livestock (Ragg *et al.* 1995b; Walker *et al.*, 1993; Ragg and Walker, 1996). The association appears, however, to be unrelated to the abundance of ferrets, either infected or in total. Ferrets, other wildlife and domestic stock in the same area have been found to share the same or very closely related *M. bovis* restriction endonuclease types (Atkinson and Cowan, 1994; de Lisle *et al.*, 1995; Ragg *et al.*, 1995a; Lugton, 1997).

One interpretation of the field evidence is that the ferrets, probably in conjunction with other wildlife, have been directly transmitting infection to stock in the same area. An alternative interpretation is that the ferrets and stock have both been infected by a common source such as possums. As yet there is insufficient data to assess the validity of these alternative explanations.

It would seem that tuberculous ferrets are potentially capable of infecting cattle and deer, the most plausible mechanism described by Sauter and Morris (1995a), being through direct investigation of a moribund animal by bold and curious individuals. However their study found the intensity of investigation to be low, and hence transmission likely to be uncommon.

One of the most important issues currently requiring clarification is whether the ferret should be regarded as a feral reservoir or maintenance host of *M. bovis* or simply a spillover host. Although it has been shown that ferrets are capable of becoming infected with *M. bovis* and will die from the disease (Dunkin *et al.*, 1929; Symmers *et al.*, 1953), it is still uncertain whether they can transmit infection amongst themselves, either pseudo-vertically or horizontally, at a sufficient rate to maintain the disease in the ferret population without regular re-introduction by cross-infection from other species. Resolution of this issue is required urgently so that sound species management strategies can be designed. This study investigated potential transmission mechanisms of tuberculosis amongst ferrets.

The information presented in this paper complements that of a companion paper (Lugton *et al.*, 1997), and in some instances provides additional detail on pathology which is more pertinent to discussion on the routes of infection.

Materials and Methods

Two hundred and twenty-eight ferrets were collected from eight areas in the North and South Islands between 1994 to 1996. Details on these ferrets, including the area of origin, necropsy and data recording procedures, collection of urine, faeces, tracheobronchial lavage fluid, pharyngeal swabs and tissue samples, bacteriology and histopathology have been described in the previous chapter.

The association between disease status, area of origin of the ferrets, sex, and age was investigated with the use of unconditional logistic regression, using the statistical software package SPSS version 7 (SPSS Inc., Chicago). The animals

were categorised as diseased if *M. bovis* was isolated from the lymph node pool, or if acid-fast bacilli (AFB) were visible in histological sections, or if characteristic liver granulomas were present. The independent variables investigated were age (months), sex, the interaction term of sex and age, and the site from which the ferrets were derived (categorised as either having, or not known to have tuberculous possums in the immediate vicinity). Relationships between diseased superficial lymph node pairs on opposite sides of the body were investigated using contingency table analysis. Contingency table analyses have been performed within the program Epi Info version 6.02 (Centers for Disease Control and Prevention, Atlanta). Where appropriate, 95% confidence limits have been presented with results.

Results

Culture or histopathological results are available for 220 of 228 ferrets examined. Seventy-three ferrets were classified as diseased (32%). In three ferrets, diagnosis was based on histological lesions consistent with tuberculosis, and in all other cases *M. bovis* was isolated. The sample was composed of 128 males (including 35 juveniles) and 98 females (including 28 juveniles). One cannibalised animal was not categorised by sex and one other juvenile male, diagnosed tuberculous by pharyngeal swab culture, was captured and released with a radio-collar, but not recovered for necropsy.

Prevalence of tuberculosis

One group of ferrets was derived from areas in which possums were recognised as an important host of tuberculosis, including (number of ferrets in brackets): Castlepoint and the Eastern Wairarapa (19), and Lake Ferry (5). Tuberculosis was diagnosed in 23 of these ferrets, giving an apparent prevalence of 0.96 (0.88-1.00). The majority of ferrets examined, however, came from areas in which tuberculous possums had been found at only low abundance, and included: Alexandra (6), North Canterbury (137), Featherston (18), Hohotaka (27), Table Hill (14), and Manakau (1). Tuberculosis was diagnosed in 50 of of these 195 ferrets, giving a point prevalence of 0.26 (0.14-0.38).

The estimated ages of all ferrets were distributed lognormally, with a median value of 7.0 months and ranging between 2 and 41 months. Of the 57 juveniles examined 12.3% were diseased, compared to 31.1% of 103 ferrets between 6 and 12 months,

and 56.9% of 58 ferrets over 12 months of age. No tuberculosis was diagnosed in any of 35 ferrets under 4 months old.

Relationships among disease status, origin, age and sex are presented in Table 4-I. The results show that ferrets derived from areas where tuberculosis was prevalent in the possum population are 99 times as likely ($p < 0.001$) to have tuberculosis as those from low prevalence areas. There is a significant increase in risk with age ($p < 0.001$), such that for each 6 month age increment there is a 2.8 times greater risk of being infected, and that this age related rise in prevalence is more pronounced in females ($p = 0.03$). Although not significant ($p = 0.22$), there was an indication that males are 1.6 times as likely to be diagnosed tuberculous as females.

Table 4-I. Logistic regression model examining the effect of area of origin, sex and age on the prevalence of tuberculosis (n = 217)

Variable	Coefficient	SE	p-value	Odds ratio (95% CI)
Intercept	-2.87	0.40	< 0.001	
Origin (hi Tb) ^a	4.60	1.08	< 0.001	99.18 (12.02-818.3)
Sex (male)	0.49	0.40	0.22	1.63 (0.75-3.54)
Age	0.17	0.03	< 0.001	1.18 (1.11-1.26)
Sex*Age	-0.07	0.03	0.03	0.93 (0.88-0.99)

Deviance = 188.5, $\chi^2_4 = 87.3$, $p < 0.001$.

a areas with a high prevalence of tuberculosis in possums.

Forty of 175 stomachs (22.8%) examined contained some food, and of these five showed evidence of possum consumption, and two of hedgehog. The stomachs of two of 17 females, and seven of 23 males, contained carrion. The odds of males showing dietary evidence of consuming carrion was 3.3 (0.5-36.3) times greater than that of females, although the observed effect was not significant (Fisher's exact 2-tailed test $p = 0.26$), probably due to the low number of animals in the analysis.

Histopathological findings

A consistent and highly significant association existed between tuberculous lesions (TbLs) in one peripheral node and the matching node on the opposite side, except for the subiliac nodes, for which there were insufficient numbers examined to establish an association (Table 4-II).

Table 4-II. Comparison of the frequency of bilateral lesions in lymph node pairs at selected sites in 54 ferrets

Lymph node	p - value	Odds ratio (95% CI)
Retropharyngeal	< 0.001	14.7 (3.0-81.3)
Caudal cervical	0.005	2.7 (1.4-5.8)
Superficial axillary	0.003	6.6 (1.5-30.3)
Deep axillary	0.005 ^a	10.2 (1.6-65.6)
Popliteal	< 0.001 ^a	11.6 (2.2-66.3)
Inguinal	< 0.001 ^a	24.2 (3.7-180.5)
Subiliac	0.51 ^a	2.3 (0.0-58.1)

a Fisher's exact 2-tailed test p-value.

There was a significant association ($\chi^2_1 = 4.7$, $p = 0.03$) between lesions in the retropharyngeal and the ipsilateral caudal cervical lymph node, the relative risk being 1.7 (1.1-2.6). A highly statistically significant association ($\chi^2_1 = 21.9$, $p < 0.001$) between lesions in the superficial axillary node and the ipsilateral caudal cervical lymph node also existed. The strength of this relationship was twice as large as that associated with the retropharyngeal lymph node (relative risk $1.9 < 3.2 < 5.4$).

Debris (such as hair shafts and unidentified foreign bodies) was found in the lymph nodes draining the skin in 51% of ferrets. These were most common in the caudal cervical and superficial axillary nodes, but also occurred in the deep axillary, popliteal, inguinal and subiliac lymph nodes. The risk of finding debris in a peripheral lymph node was 1.2 (0.9-1.5) times greater for diseased as for non-diseased animals. This difference was not, however, significant ($\chi^2_1 = 1.09$, $p = 0.30$). There was also 1.2 (0.8-1.8) times greater risk for males having debris, although this difference was again not significant ($\chi^2_1 = 1.06$, $p = 0.30$). Mature animals had a 1.2 (0.9-1.5) times greater risk of debris accumulation in peripheral nodes compared with juveniles, although this difference was also not significant ($\chi^2_1 = 1.07$, $p = 0.30$). There was a slightly greater, although non-significant risk, of finding debris in those animals thought to have been infected percutaneously versus those infected via the alimentary tract (odds ratio $0.3 < 1.8 < 10.8$, Fisher's exact 2-tailed test $p = 0.70$). No retropharyngeal lymph nodes were found to contain debris.

Portals of disease entry

In 16 of the 54 diseased ferrets, lesions were widely disseminated, with extensive necrosis in several sites, so that it was not possible to identify a primary lesion or probable portal of entry. In 38 animals, the probable primary lesion could be identified, either because only one site was involved or because the lesion in one site was advanced with extensive necrosis while lesions at other sites were small, less advanced and without extensive necrosis, or because isolation of *M. bovis* from pooled lymph nodes made it likely that the infection was present in either the retropharyngeal or jejunal lymph nodes. From these observations 30 animals (79%) appeared to have had the primary lesion in lymph nodes draining the alimentary tract (including the retropharyngeal node and oropharyngeal tonsils). Three of these 30 cases (8%) also had primary sites involving both the retropharyngeal and caudal cervical or superficial axillary lymph nodes, and thus could possibly have been first infected in the cervical area as an alternative to the alimentary tract route. The remaining eight of 38 (21%) appeared to be infected via sites draining to the peripheral body lymph nodes, the most likely route being percutaneous. These primary sites included three cases of subiliac node infection, two caudal cervical, one superficial axillary and two cases of combined superficial axillary and caudal cervical node infection. The odds of a mature ferret apparently being infected in these sites was 2.6 (0.3-128.9) times that of a juvenile, but this difference was not significant (Fisher's exact 2-tailed test $p = 0.65$).

Routes of excretion

Mycobacterium bovis was isolated from 15 of 64 (23.4%) pharyngeal swabs. Histopathological data was available on 51 of these ferrets, including twelve of the swab-positive animals. There was a positive but non-significant association between head-associated lesions and isolates of *M. bovis* from the pharynx (Fisher's exact 2-tailed test $p = 0.17$, odds ratio $0.7 < 3.9 < 40.0$).

Five ferrets had lesions identified in the oropharyngeal tonsils and isolates of *M. bovis* from pharyngeal swabs were obtained in four of these cases. Three of four ferrets with positive swabs had AFB visible on tonsillar sections, whereas the one case with a tonsillar lesion and a culture-negative swab had no AFB visible in the lesion. Pharyngeal swab-positive animals were significantly more likely to have tonsillar lesions (Fisher's exact 2-tailed test $p = 0.009$, odds ratio $1.5 < 19.0 < 959.0$).

Four of 64 ferrets (6%) sampled were found to have AFB in tracheobronchial lavage fluid. Three of the four animals had pulmonary or thoracic lesions which could definitely be attributed to tuberculosis, and the fourth had “lipid plaques”. Animals from which culture-positive lavage was obtained were more likely to be found with tuberculous thoracic lesions, but the relationship was not significant (Fisher’s exact 2-tailed test $p = 0.32$, odds ratio $0.3 < 3.9 < 212.5$). All four ferrets also had advanced-stage disease and pharyngeal isolates of *M. bovis*.

Positive cultures of *M. bovis* were obtained from faeces or colonic swabs from ten of 63 ferrets sampled (16%). Histopathological examination was performed on 50 of the 63 ferrets from which these swabs were obtained. All ten with *M. bovis* isolates had tuberculous liver and bowel-associated lesions. Nine of these had greater than ten TbLs; and the other one had eight TbLs: This association between faecal *M. bovis* isolates and bowel-associated lesions was statistically significant (Fisher’s exact 2-tailed test $p = 0.044$). Of the animals with bowel-associated lesions, there were six ferrets with tuberculous intestinal lesions. *Mycobacterium bovis* was isolated from faecal cultures taken from four of these six ferrets (odds ratio $1.4 < 12.7 < 156.1$; Fisher’s exact 2-tailed test $p = 0.011$).

Mycobacterium bovis was also isolated from two of 29 urine samples (7%). Histological results were available for only one of these cases. This ferret had necrotic lesions with AFB in both kidneys. The other urinary isolate came from an animal with advanced disease, but no gross kidney lesions. A culture-negative urine sample came from another ferret which had granulomatous kidney lesions without AFB.

One of eight mammary glands taken from infected females yielded *M. bovis* when cultured (12.5%). The infected mammary gland contained inspissated milk, and was from a ferret with “early” disease (chapter 3) which had necrotic lesions of the jejunal lymph node, and granulomas in the lung, liver, one popliteal and one bronchial lymph node.

Draining tuberculous sinuses (Figure 4-1) were seen in two ferrets only, one involving a popliteal and the other an inguinal lymph node. Large numbers of bacilli are likely to be excreted in the discharged necrotic exudate, but this was not investigated bacteriologically.



Figure 4-1. Draining sinus from a tuberculous inguinal lymph node. These are an uncommon finding, but are likely to produce environmental contamination and increased risk of intraspecific transmission.

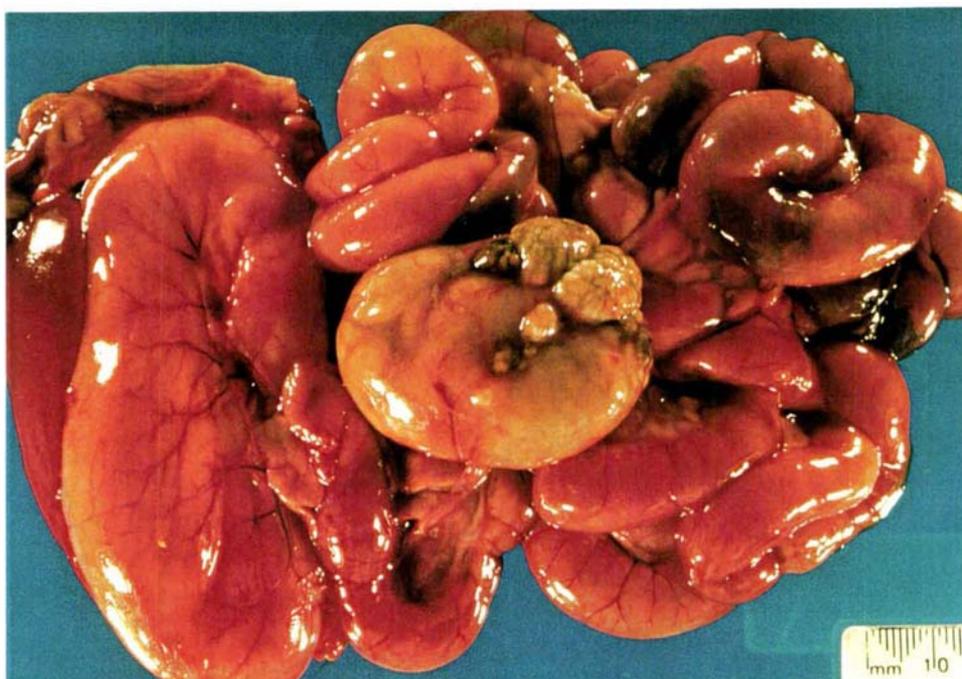


Figure 4-2. Grossly enlarged jejunal lymph node containing a large volume of “milky” necrotic contents. Severe lesions such as this are associated with advanced disease and possible bacillary excretion by several routes.

Discussion

The exceptionally high prevalence of tuberculosis, approaching 100%, found in ferrets in areas with an entrenched wildlife reservoir of *M. bovis* has hitherto been unreported. The only ferret not to have tuberculosis in this group of 24 animals was a 2-month-old road casualty. The other group of ferrets (26% prevalence), selected from areas with minimal or no recognised possum tuberculosis, included ferrets from known endemic areas for bovine tuberculosis (e.g. Hohotaka and Featherston), but both possum abundance and the prevalence of tuberculosis at these sites were low at the time of investigation (J. McKenzie, pers. comm.; Caley, 1995b). The significant difference in the prevalence of disease between these two categories suggests that the availability of tuberculous carrion may be an important cause of the disease in ferrets. A positive relationship between the abundance of possums and the prevalence of tuberculosis in ferrets has been established in another study (P. Caley, unpublished data).

The increasing prevalence with age, also reported by Ragg *et al.* (1995a), and the absence of disease in ferrets under 4 months of age indicates that exposure and infection before weaning at 6-8 weeks, if they occur, appear to be rare events, even though solids are first consumed at about 20-30 days of age (Lavers and Clapperton, 1990). This may be due to the nursing mother not feeding carrion to her offspring, or could be simply due to an inability to bring large carcasses back to the den site. Weanling ferrets may also need time to develop the strength or skill to break into a possum carcass. Only two juvenile ferrets were diagnosed with advanced disease, and both of these came from the areas where tuberculosis was prevalent in possums, supporting the hypothesis that infection is principally related to ingestion of tuberculous carrion. Possums and hedgehogs, both species which can be infected with *M. bovis*, have been shown to be eaten frequently by ferrets in several areas in New Zealand (Roser and Lavers, 1976; Robertson, 1976; Smith *et al.*, 1995), and often occur as dietary components in over 10% of animals sampled. The prevalence of infection in ferrets clearly rises with age, and this may indicate that the disease is capable of running a prolonged course, as well as reflecting that as a ferret ages, the likelihood that it will have consumed tuberculous material will increase. The higher proportion of infected adults versus juveniles is also in accord with the findings of Ragg *et al.* (1995a). This contrasts with the possum, in which infection occurs by both the pseudo-vertical and horizontal routes, and duration of disease is probably

shorter. As a consequence, prevalence in the possum shows no increase with age (Jackson *et al.*, 1995a).

Male ferrets appear to have a slightly greater risk of acquiring infection than females. Although the difference was not statistically significant in this study, it is likely to be a true reflection of the state of nature, as the same disproportion was observed by Ragg *et al.* (1995a). Male stoats (*Mustela erminea*) have been shown to scavenge more carrion, including possums, than females (King and Moody, 1982; Murphy and Dowding, 1994; Murphy and Dowding, 1995;). This behavioural difference may also occur in ferrets, as they too also commonly eat carrion (Roser and Lavers, 1976; Mills, 1994); our limited dietary data also supports these observations. This sex difference in diet offers one plausible explanation for the higher prevalence observed in males. Possum consumption can still be implicated as a cause of tuberculosis even in localities where tuberculous possums are not found, as ferrets have been known to travel up to 2 km per night, and dispersal movements of 50 km in less than 4 months have been recorded (Mills, 1994). Possum carcasses which have been scavenged by ferrets often have the eyes, scrotum and pouch young removed initially, and the carcass then entered by the pouch with the inguinal area eaten first (unpublished observations). Consumption of this area of the carcass must entail the ingestion of the inguinal lymph nodes, a predilection site for tuberculosis in the possum (Jackson *et al.*, 1995). Cannibalism of young in the nest is a recognised problem of domestic ferrets, but there have been no occurrences of ferret remains reported in New Zealand dietary studies. One juvenile in this series which died in a leg-hold trap was thought to have been cannibalised by litter mates. G. Norbury (pers. comm.) has also seen one of two ferrets trapped in a mine shaft eaten by the other, but none of 75 radio-collared ferrets which died in the field have showed evidence of cannibalism, nor were ferret remains found in any of more than 1000 ferret scats examined.

Over half the ferrets had microscopic debris in peripheral lymph nodes. This foreign material is probably indicative of skin wounding, with introduction of hairs and other wound contaminants at the site. Although none of the statistical associations of debris occurrence were significant, and the associated increased risks small, the pattern of more older and male ferrets being involved fits the hypothesis that most of the injury is from bite wounding through intraspecific strife. Ferrets

are often found with multiple puncture wounds, especially during the breeding season as a result of aggression amongst males and the traumatic mating ritual in which the female is repeatedly bitten and grasped about the neck. G. Norbury (pers. comm.) has noted up to 40% of ferrets with bite wounds during the spring breeding season, the vast majority of these wounds occurring in males. Ragg *et al.* (1995a) also reported that the majority of ferrets captured during the breeding season had open wounds apparently associated with fighting and mating. The significant association between infection in opposite pairs of peripheral body nodes is also explicable in terms of bite wounding, as bites from a proportion of infected ferrets are likely to contaminate wounds, inflicted on both sides of the body, with *M. bovis*. Early generalisation of the disease, with spread to peripheral lymph nodes, may also be responsible for this bilateral involvement.

An anatomical link between both the retropharyngeal and the superficial axillary lymph nodes and the ipsilateral caudal cervical node has been described by Shibata *et al.* (1988). Efferent lymph drainage to the caudal cervical nodes from these sites is likely to be responsible for the statistically significant associations observed between these tissues. The lower risk of lesions occurring in the caudal cervical node in association with infection in the retropharyngeal node may infer that this drainage pathway is less constant than that from the superficial axillary. Overall, it can be concluded that the percutaneous route of transmission is significant, but much less common than the oral route.

Gross lesions were most prevalent in the jejunal lymph node (Figure 4-1) (Lugton *et al.*, 1997), being found in nearly half of the infected animals. No other site of gross lesions approaches the same frequency of occurrence, the nearest being the retropharyngeal lymph node with a prevalence of 0.21. This high frequency of jejunal lesions has been found in previous investigations of the disease in ferrets (Ragg *et al.*, 1995c), and provides strong evidence for the bulk of the infections having an alimentary tract origin. Indeed, 79% of the ferrets in which a primary lesion site could be reasonably determined showed evidence of infection from the alimentary tract, with the remainder seemingly infected from bite wounds to the neck and body. Although not a significant association, there appeared to be an increased risk of percutaneous infection in mature animals, consistent with expectations of increased bite wounding with the attainment of sexual maturity.

About one quarter of the infected ferrets had evidence of oral excretion of bacilli, apparently unrelated to the stage of the disease. The true prevalence of bacillary excretion at any site is likely to be higher than was recorded in the current study, due to the low sensitivity of recovery of small numbers of *M. bovis* in the presence of contaminants. Oral excretion was related to infection around the head, although the data failed to show a statistically significant association. The failure to show a significant association may have arisen due to the reduced sensitivity of retropharyngeal node examination following removal of half to two thirds of each retropharyngeal node, including necrotic areas, for culture. Walker *et al.* (1993) also isolated *M. bovis* from a pharyngeal swab taken from a ferret with a retropharyngeal lesion.

Despite the low number of cases, there is clearly a strong association between having tuberculous tonsillitis and recovery of *M. bovis* from the oral cavity. The process of swabbing the pharynx is likely to abrade the epithelium, and so dislodge mucosal macrophages, as well as soak up bacilli in oral secretions. The high proportion of infected retropharyngeal nodes was probably directly related to acquisition of infection by the tonsil, with direct extension by lymphatic drainage to the ipsilateral retropharyngeal lymph node. The lymphoepithelium overlying the tonsils contains M-cells which actively endocytose bacilli and other antigenic material (Payne and Derbyshire, 1963; Fujimura, 1986), and also permit the movement of lymphoid cells through the epithelium to the mucosal surface (Perry, 1994; Belz and Heath, 1995a). This specialised epithelium can thus provide a means both of entry and potential excretion for mycobacteria resident in the tonsil. Any tuberculous lesion disrupting the tonsillar epithelium could also discharge bacilli to the oral cavity, though no such lesions were observed. The retropharyngeal nodes seem unlikely to be infected by bite wounds, as injuries to the head have not been seen or reported, and no evidence of debris was found in any of these nodes. Injury to the oral mucosa may also allow uptake of bacilli to the lymphatic system, but no cases of oral wounding were noted.

The absence of necrotic or extensive pulmonary lesions does not favour the excretion of bacilli from the respiratory tract, and in this study respiratory shedding was restricted to a few of the animals with advanced disease, typically having lesions containing AFB in the lungs or draining lymph nodes. Aspiration of blood

into the trachea (following a blow on the head) could possibly have been responsible for introduction of bacilli into the airways in some cases, as each of these ferrets also had *M. bovis* recovered from pharyngeal swabs. Tracheal isolates of *M. bovis* have also been collected in two instances by Cowan (1994), both from ferrets with extensive gross lesions.

Intestinal excretion of substantial numbers of bacilli appears most likely from animals with advanced disease, and this observation is supported by Cowan (1994) who also isolated *M. bovis* from rectal swabs of two ferrets with advanced disease. There is a very high risk for faecal shedding from lesions of the intestinal tract, even though none were seen involving the mucosa. Symmers *et al.* (1953) found numerous non-ulcerated caseous foci in the gastro-intestinal mucosa in one naturally infected animal, and it is possible that a few similar lesions may have been overlooked in the present series. Ulceration of the mucosa, especially involving Peyer's patches, appears to be associated with ingestion of bacilli-laden respiratory secretions (Jennings, 1949), and although occurring in other species, is an unlikely sequel to infection in the ferret. The liver is also potentially capable of releasing bacilli into the intestine via biliary drainage. Urinary excretion also seems to be only associated with advanced stage disease and kidney involvement. Open draining sinuses were identified in two ferrets in this study and also in six of 98 infected ferrets by Ragg *et al.* (1995c).

Of the routes of bacillary excretion, only oral shedding, mammary involvement and draining fistulae would seem likely to play a significant role in the intraspecific transfer of infection. Ferrets use latrines near their den sites to deposit faeces and also mark their territory with anal gland secretions and urine (Blandford, 1987): These are likely to be sniffed but not otherwise investigated by other ferrets. Respiratory excretion is uncommon, and as ferrets commonly thought to lead solitary existences for most of the year, is probably of little consequence. Mammary infection has been reported in mink (Pulling, 1952) and was found in one of eight sets of glands cultured in this study. It seems therefore that pseudo-vertical transmission, by whatever means, is possible but likely to be a rare event as no weanling ferrets have been found infected. Draining fistulae, although occurring infrequently, and only found in advanced cases, may be responsible for infrequent contamination of den sites, and are likely to heavily contaminate the skin surface,

enabling transfer of infection to assailants or inquisitive stock. The moderate prevalence of oral shedding, and its occurrence in some cases throughout much of the period of infection, has important implications for the transfer of infection via intraspecific fighting, although the data on primary lesion sites suggests that no more than one in five infected ferrets may have become infected in this manner. The association between the presence of debris and the apparent route of infection does not add much support to the hypothesis that bite wounding is an important mode of transmission.

Hind limb paralysis has been described as a feature of the disease in two ferrets by Symmers *et al.* (1953). This phenomenon of hind limb paresis or paralysis has also been observed in natural cases of the disease in New Zealand, both in the field and in captivity. This terminal disability could in part be responsible for over 60% of dead ferrets being found exposed on the surface (G. Norbury, pers. comm.), and the inco-ordinated movements are likely to attract the attention of inquisitive stock. One dead ferret which was submitted to us was destroyed whilst exhibiting hind limb paralysis. This ferret had early stage lesions, and appeared to be dying from starvation. No lesions of the aortic endothelium, or any associated thrombi could be found. Examination of the central nervous system (CNS), and musculature also failed to demonstrate lesions. Several other ferrets with advanced disease were also examined for the presence of aortic or CNS lesions, with negative results. This is consistent with the negative CNS findings of Symmers *et al.* (1953), who did, however, find aortic endothelial damage and mild adductor muscle lesions. Hind limb paresis has also been reported in mink (*Mustela vison*) (Head, 1959) which had been ill from other causes. Hind limb paresis may thus be related to physiological disturbances, in the terminal stages of a number of conditions in this genus, rather than being a direct consequence of tuberculosis.

The one ferret which was fitted with a radio-collar was known to have survived for 1 year with tuberculosis before radio contact was lost. Two ferrets which died from tuberculosis were estimated to be 9 and 21 months old, and a road casualty with severe disease 10 months of age. Three other animals with very advanced disease (over 20 TbLs) were estimated to be 4, 11 and 36 months old, respectively. In experimental infections had one animal remained alive for 6 months after an intramuscular inoculation of 5×10^4 bacilli (Symmers *et al.*, 1953), and one animal

died 7 weeks after subcutaneous inoculation of 1.0 mg of *M. bovis* (Dunkin *et al.*, 1929), and another was killed 4.5 months after being administered 1.0 mg orally, which showing evidence of early generalised disease (Dunkin *et al.*, 1929). By extrapolation from other species, experimental infections with high doses of bacilli may be expected to have far shorter duration than field infections (Jackson *et al.*, 1995a). From this it is hypothesised that the time of survival after infection in ferrets is variable, probably ranging from several months in a few, to in excess of a year in many. The two animals we found with only one or two necrotic peripheral node lesions, but without liver granulomas, provide evidence that some ferrets can mount an effective immune response and localise infection. The average life expectancy of ferrets is only short, as Norbury and Heyward (1995) have found that about half of the population is replaced each year. The average life span of male polecats (the wild ancestor of ferrets) in Britain has similarly been estimated at only 8.1 months (Blandford, 1987). Thus it must be expected that many ferrets infected with tuberculosis will die from other causes before the disease process produces any morbidity. Given that infection is unusual until a ferret reaches at least 6 months of age, the data presented here strongly suggests that most ferrets become infected by eating tuberculous carrion over the remainder of their life, with the period prevalence in ferrets being putatively linked to the amount of tuberculous carrion available. The prolonged course of the disease may also contribute to a high point prevalence, even in areas where tuberculous carrion is scarce.

Strain differences in pathogenicity may also exist in the various *M. bovis* restriction endonuclease (REA) types. The very severe disease involving caseous lung lesions, pleuritis, gross hepatic lesions and massively enlarged spleens described in ferrets in the British studies have not been noted in our cases, and in the North Island it has been difficult to find lesioned cases in areas where the central North Island REA types are known to be endemic. Strain differences are, however, unlikely to account for the variation in prevalence of tuberculosis infection between different geographic areas, unless South Island strains have a markedly reduced ability to establish infection in ferrets.

The absence of significant respiratory excretion in tuberculous ferrets means that for susceptible domestic stock to become infected by investigating a sick ferret, that they almost certainly will need to have intimate physical contact, probably similar

to that described by Boyd (1996). Ferrets with discharging fistulae may thus be infectious for stock which lick the ferret, and others may become infected by being bitten by a frightened and aggressive creature. If these hypotheses are correct, then reactor animals infected by ferrets are more likely to have lesions in the full range of head lymph nodes than livestock infected by the respiratory excretions of possums. Given the low density of ferrets ($< 10/\text{km}^2$), especially moribund tuberculous ferrets, and the general lack of enthusiastic exploratory behaviour by cattle and deer which come close to them (Sauter and Morris, 1995a), infectious encounters are likely to be infrequent. This could offer an explanation for the low incidence of reactors in areas where ferrets are thought to be responsible for infecting livestock (Livingstone, 1996), as could a low density of tuberculous possums in these areas.

It is clear that horizontal transmission in ferrets can, and probably does, occur through fighting and mating associated injuries. Transmission through cannibalism and pseudo-vertical infection may also be possible, but evidence still remains elusive, and these routes are unlikely to contribute substantially to the prevalence. If infection were acquired from social contact with other ferrets, the prevalence would be density-dependent, but Ragg *et al.* (1995a) has shown this not to be the case. Historically, there have been troublesome numbers of ferrets for many decades (Lavers and Clapperton, 1990), and these animals will have had ample access to carcasses of tuberculous cattle throughout periods when the prevalence in cattle was high and clinical cases abounded. Ferrets have even been known to shelter inside carcasses of dead deer and cattle (Hammond, 1996). Despite the exposure of ferrets in what are now considered non-endemic areas, no apparent reservoir of tuberculosis has developed in the ferret populations.

Tuberculosis in possums is characterised by low point prevalence and high period prevalence, due to the apparent short survival time of diseased possums. This makes infected possums surprisingly difficult to locate, considering the proportion which become infected during their lifetimes. In contrast, ferrets have high point and annual prevalence, indicating a disease of substantial duration. It would seem that ferret populations sample tuberculous carrion at a steady rate, as infection increases steadily with age, and does not show marked local spatial heterogeneity. The evidence suggests that this carrion is typically possums, although it may include other species and occasionally other ferrets.

There is, at present, inadequate evidence to conclude that the flow of infection through intraspecific transmission pathways will be enough to ensure maintenance of the disease in ferret populations without repeated introduction of *M. bovis* from other food-borne sources. The evidence presented in this paper, and the review of the current situation, does not support the notion of the wild ferret being another reservoir host of tuberculosis in New Zealand.

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The PhD candidate was responsible for the study design, execution of the vast majority of the necropsies and collection of samples for bacteriology, analysis of data and preparation of the manuscript. Gary Wobeser (on leave from the Canadian Co-operative Wildlife Health Center, Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada.) performed the histopathological examinations, and Peter Caley (Landcare Research) provided a large number of ferrets collected from his North Canterbury study site.

CHAPTER 5

NATURAL INFECTION OF RED DEER WITH BOVINE TUBERCULOSIS¹

¹ Published as I. W. Lugton, P. R. Wilson, R. S. Morris, J. F. T. Griffin and G. W. de Lisle, (1997) New Zealand Veterinary Journal, 45, 19-26.

Abstract

Six bovine tuberculosis-free red deer hinds were introduced in October 1993 to a 1.8 ha enclosure, within a larger field study site known to contain tuberculous possums, and kept there for 9 months. A *Mycobacterium bovis*-infected possum was found in the vicinity of the deer enclosure three weeks after the introduction. Subsequently, a further eleven infected possums were found in the area. The deer were monitored by repeated composite antibody detection ELISA and lymphocyte transformation assays for tuberculosis, interpreted in parallel, by skin testing and by routine culturing of samples collected from potential excretion sites. Lymphocyte transformation assay evidence of *M. bovis* infection in four hinds was first observed 4 months after introduction. One other hind became bovine tuberculin lymphocyte transformation assay positive in the 5th month. Positive or equivocal bovine reactivity remained evident at most test episodes. A comparative cervical skin test performed in July 1994, shortly before slaughter, was positive in these five hinds. *Mycobacterium bovis* was recovered off swabs from the oropharyngeal tonsils of two hinds during routine sampling. Detailed necropsy of the six deer revealed a single typical tuberculous lesion in only one, but culturing of various tissue specimens ascertained that the five blood test and comparative cervical skin test positive animals were all infected. *Mycobacterium bovis* was cultured from the oropharyngeal tonsils of four and medial retropharyngeal lymph nodes of two of the deer with no typical gross lesions. Six additional tuberculosis-free hinds were introduced to the enclosure in April 1994 and kept there for 12 months. Four of these animals showed a positive lymphocyte transformation assay response to *M. bovis* after 9 weeks, but no significant reactivity thereafter.

Concurrent observational studies suggest that five of the first six deer probably became infected through close inspection and investigation of the tuberculous possums, although the possibility of deer to deer transmission cannot be totally excluded. The likely deer-possum contact, and thus exposure to *M. bovis* was related to the curiosity and social ranking of the hinds. The second group appear to have had transient exposure to *M. bovis*, possibly caused by direct contact with the infected hinds introduced earlier. This group never showed any curiosity toward, or interaction with, possums during the periods of observation.

Introduction

Wild and farmed red deer (*Cervus elaphus*) are believed to be important in the epidemiology of tuberculosis in New Zealand. They have been implicated in the spread of infection to possum populations (*Trichosurus vulpecula*) (Livingstone, 1988; Mackereth, 1993) and it is widely accepted that they commonly acquire infection from tuberculous possums (Jackson, 1995; Morris and Pfeiffer, 1995). Deer are also thought to be probable reservoir hosts of *Mycobacterium bovis* (Morris and Pfeiffer, 1995).

Behavioural observations of possum-deer interactions, including details of the same deer used in this study, have been reported elsewhere (Sauter and Morris, 1995a; Sauter and Morris, 1995b). Bolder, socially dominant deer would sniff, lick, bite and strike at possums sedated to simulate the effect of advanced tuberculosis infection. Large numbers of *M. bovis* organisms are excreted by terminally ill tuberculous possums (Jackson *et al.*, 1995b). Thus behavioural interactions should expose deer to the risk of oral and/or respiratory route infection from tuberculous possums.

Lesions in tuberculous deer most commonly involve the medial retropharyngeal lymph nodes (Hathaway *et al.*, 1994; Mackintosh and Griffin, 1994). These lymph nodes drain the tonsillar tissues of Waldeyer's ring, and it is probable they become infected after primary involvement of tonsillar tissues which have been shown to actively take up mycobacteria (Payne and Rankin, 1961; Fujimura, 1986; Momotani *et al.*, 1988). Experimental studies have shown that artificial infection of the oropharyngeal (palatine) tonsils in deer produces disease indistinguishable from that found naturally (Mackintosh *et al.*, 1993), whereas infection by the intravenous, subcutaneous or intra-tracheal routes does not (Brooks, 1984; de Lisle *et al.*, 1985). While these indirect observations suggest that the oral route may be the primary means of establishing natural *M. bovis* infections in deer, no direct evidence has been reported.

This study was established to evaluate the hypothesis that the natural mode of transmission of *M. bovis* from possums to deer was via inhalation or ingestion as a consequence of direct contact with tuberculous possums.

Materials and Methods

Site

A deer enclosure was erected on part of a 21 ha bush and pastoral site on a Wairarapa sheep and beef property. This site was one in which tuberculosis was present in possums, and which has been the subject of a longitudinal study known as the "Castlepoint Study", reported elsewhere (Pfeiffer, 1994; Jackson 1995). This site allowed the monitoring of deer in an environment where tuberculous possums were regularly trapped and closely observed.

The deer used for this study were introduced to a small deer-fenced area on the lower section of the study site in which moribund and dead tuberculous possums had been found previously. Three deer paddocks were sited on a grassed, gently sloping area, which had scattered manuka trees on the periphery and which was dissected by a small ephemeral stream. The most elevated paddock of about 0.8 ha, which had the most manuka and the greatest likelihood of possum contact, was where the deer in Group 1 (below) spent the majority of their time. This enclosure contained a small pen and a deer handling darkroom topped by a 5 m high observation tower used for the behavioural observations (Sauter and Morris, 1995b). The lower paddocks of about 0.5 ha each were utilised significantly only after the arrival of the second group of deer. On the northern side of the study site, adjacent to the most elevated deer paddock, five to eight steers were depastured. These were skin tested every 3 months, with reactors removed and subjected to detailed necropsies and culture of suspicious lesions.

Animals

Six non-pregnant rising 2-year-old red deer hinds from an accredited tuberculosis-free closed herd, located away from declared movement controlled areas, were introduced to the study site on 20 October 1993 (Group 1). All deer tested negative to the parallel interpretation of a combined antibody detection ELISA and lymphocyte transformation assay (LTA), commonly known as the blood test for tuberculosis (Griffin *et al.*, 1994) or BTB, 2 months prior to movement. Another six non-pregnant hinds, aged 3-5 years old, from the same property (Group 2), were introduced to the study site on 9 April 1994 after a negative BTB on 18 March 1994. The source herd remained tuberculosis-free at a routine surveillance test in

June 1995. All deer were health checked, condition scored, weighed, individually identified, drenched with anthelmintic and vaccinated against clostridial diseases immediately prior to shipment to the trial site.

Observations

Group 1

The first six hinds had their girths measured, their condition scored, were physically examined and had a blood sample collected for the BTB initially every 2 months. The timing of these examinations was later varied to as little as 10-14 day intervals when evidence of *M. bovis* infection became apparent, according to the schedule in Figure 5-1. Parallel interpretations of the LTA and the ELISA test results, were according to the criteria of Griffin *et al.* (1994). On 15 May 1994, 0.1 ml of bovine tuberculin (Tuberculin PPD [Bovine], 1 mg/ml, Central Animal Health Laboratory, Wallaceville Animal Research Centre) was injected intradermally, in an attempt to stimulate an antibody response. A comparative cervical test (CCT) as described by Corrin *et al.* (1993) was applied on 11 July 1994, using 0.1 ml each of avian tuberculin (Tuberculin PPD [Avian], 0.5 mg/ml, CSL Ltd) and bovine tuberculin as above.

The nasal cavities and both oropharyngeal tonsillar fossae of the hinds were swabbed, following sedation with xylazine (0.4 mg/kg i.m. Xylazine 2%, Phoenix Pharmaceutical Distributors Ltd), on 20 March and 15 May 1994. Commercial swabs containing transport medium (Transwab, Medical Wire and Equipment Co. Ltd) were used, except for the tonsillar sites, where a shortened guarded intrauterine type swab was used (Guarded Culture Instrument, K₁ 2000, Cenvet Pty. Ltd). Each tonsillar swab was guided through a sterile 40 mm PVC pipe oral speculum, aided by penlight illumination. Faecal samples were also collected from each deer by gloved digital extraction from the rectum. All samples were submitted for mycobacterial culture.

On 26 July 1994, all six hinds were killed on site by the intravenous injection of sodium pentobarbitone. Abdominal contents were removed and placed in separate bags. A faecal sample was extracted from the cut end of the rectum, and a urine sample was removed from the bladder following needle puncture. The mucosal surface of the mid-cervical section of the transected trachea was swabbed, using the transport medium swabs described above. Nasal, and tonsillar swabs, were also

collected as above. The carcasses and abdominal viscera were transported to Massey University, chilled overnight and detailed necropsies performed the following day (as outlined in Appendix I). All major lymph nodes were laid out on pre-labelled paper sheets and sliced at 2 mm intervals. Lungs were sliced to a 2 cm thickness to facilitate thorough palpation and visual examination. Any suspicious lesions were kept for culture and histopathology. In addition, four tissue pools were retained from each deer for culture, comprising a sub-sample of both the oropharyngeal tonsils, the nasopharyngeal tonsil (adenoid), a sub-sample of both medial retropharyngeal lymph nodes, and a combined sample of the caudal mediastinal, left tracheobronchial and the cranial tracheobronchial (apical) lymph nodes. Isolation of *M. bovis* from faeces, urine, and the nasal, tonsillar and tracheal swabs collected at slaughter was attempted if cultures from the submitted tissue pools confirmed the animal as tuberculous.

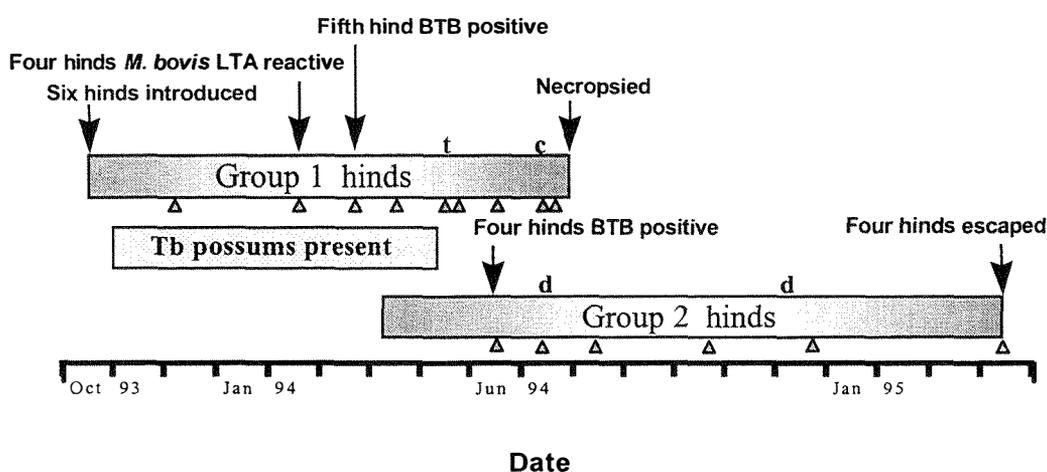


Figure 5-1. Sequence of events and observations involving tuberculous possums and deer at the study site. t = tuberculin injection; c = comparative cervical test; d = death of one hind; Δ = BTB sampling

Group 2

These deer were BTB tested 2 months after arrival, then at monthly intervals for the next two tests and less frequently thereafter (Figure 5-1). They were sedated as above and tonsillar fossae and nasal cavities swabbed on 11 July 1994. Two which died of natural causes were necropsied.

Possoms

Routine monthly cage trapping of possums as described by Pfeiffer (1994), was conducted on the study site surrounding the deer enclosure. Trapped possums were tranquillised and physically examined for signs of tuberculosis. In addition, dead possums found in the vicinity of the deer paddocks were necropsied, and lesions cultured for *M. bovis*. Sick live possums, seen in daylight, were captured by hand and examined; aspirates or swabs from lesions resembling tuberculosis were taken for mycobacteriological culture. Radio-collars were fitted to all possums suspected of being tuberculous, to enable tracking to daytime den sites and to facilitate carcass recovery.

Specimen handling

To avoid cross contamination of samples for culture, instruments were immersed in boiling water as appropriate. Tissues removed for bacteriology were stored in sterile plastic containers, and together with swabs and other samples, kept at -84 °C. Samples were later submitted to the AgResearch tuberculosis laboratory, Wallaceville Animal Research Centre, for culture. Routine culture techniques described by Buddle *et al.* (1994) were used to isolate *M. bovis* from tissue samples. Urine and faeces were cultured using the method described by Jackson *et al.* (1995b). Swabs were cultured by vortexing them in 10 ml of sterile-distilled water and then 5 ml of this solution was decontaminated by the addition of an equal volume of 0.75% w/v of cetyl pyridinium chloride. The remainder of the culture procedure for the swabs was the same as that used for tissues. Typing was performed using DNA restriction endonuclease analysis (REA) according to the technique of Collins and de Lisle (1985). The numbering of REA types was the same as that used by Collins *et al.* (1986). Formol-saline fixed tissues from culture-positive deer were submitted to the Batchelar Animal Health Laboratory, Palmerston North. Paraffin-blocked tissue prepared from this material by routine histological processing was sectioned and stained using the haematoxylin and eosin and Ziehl-Neelsen methods. Heparinised blood samples were sent by overnight courier to the Deer Research Laboratory, University of Otago, for BTB testing as described by Griffin *et al.* (1994).

Results

Association with tuberculous possums

The relationship between tuberculous deer and possums are presented in Figure 5-1, and details of tuberculous possums are presented in Table 5-I. Tuberculous possums were first found inside or within 30 m of the deer enclosures 3 weeks after arrival of Group 1 hinds. During the first 3 months on site, six tuberculous possums were known to have died outside of the deer enclosures. Of these three were found dead, and the remainder were identified as tuberculous whilst still alive. One of these possums (2841), which had a draining inguinal lesion, was known to have denned inside the deer enclosure amongst lopped manuka branches, on at least 2 occasions. This possum was the only one of the first six to be infected with REA type 10b (a new REA type, closely related to type 10), whereas the other five were infected with type 4.

Table 5-I. Details of tuberculous possums found in or near the deer enclosures

Possum	Date found	State	Survival (days)	REA type	Comments
2839	10/11/93	putrid	-	4	10 m to north, outside deer enclosure
3581	10/11/93	putrid	-	4	1 m to south, outside deer enclosure
2147	15/11/93	alive	82	4	lived to north, but died on southern side outside deer enclosure
2842	21/11/93	alive	4	4	died 30 m to west, outside deer enclosure
2841	8/12/93	alive	27	10b	denned in deer enclosure, died outside; draining inguinal lesion.
2852	14/12/93	putrid	-	4	10 m to north, outside deer enclosure
3633	14/2/94	fresh dead	-		20 m to north of deer enclosure; <i>M. bovis</i> not isolated
2266	21/2/94	alive	25	4b	denned either side outside deer enclosure, died outside; draining axillary lesion.
2958	22/3/94	fresh dead	-	4	traversed deer enclosure prior to death outside deer enclosure; draining inguinal lesion
2219	3/5/94	fresh dead	-	10b	died inside deer enclosure in higher paddock when deer absent
2401	10/5/94	alive	3	10b	seen inside enclosure with deer, died 1 m outside
2319	11/5/94	fresh dead	-	10b	died in enclosure, but carcass inaccessible inside darkroom

From mid-February 1994 a further six tuberculous possums were found. Four of these were freshly dead in locations which were inaccessible to the deer. One live possum (2266) which denned on either side of the deer enclosure, survived for nearly 1 month after clinical diagnosis. This was the only possum to be infected by type 4b (a new restriction type closely related to type 4). None of the possums necropsied at any time showed evidence of trauma which could have been attributed to deer attack.

Blood and intradermal tests

The results of sequential LTA and ELISA tests from Group 1 deer are presented in Figure 5-2, and all test results are summarised in Table 5-II. Group 1 deer all showed evidence of LTA avian tuberculin reactivity prior to introduction to the study site and at the first test after arrival, except for Hind 6 which showed an equivocal bovine tuberculin LTA response to the first on-site test. Hinds 1 and 5 were *M. bovis* LTA positive 4 months after introduction. Equivocal LTA reactions were observed in Hinds 4 and 6 at this time. Hind 3 converted to *M. bovis* LTA reactivity after the 5th month on-site and hind 4 continued to show equivocal LTA responses until an *M. bovis* ELISA positive result after tuberculin stimulation 7 months after arrival, when she became BTB positive. After initial positive BTB results, four of the hinds had a reduction in *M. bovis* LTA responses, such that test interpretations were either avian or equivocal on at least one occasion. The BTB results later reverted to *M. bovis*-positive for the same four hinds. Injection of bovine tuberculin produced a positive *M. bovis* ELISA response in three of the LTA-reactive deer after the first injection, and in four deer after the second injection. Hind 3 became ELISA equivocal and Hind 2 remained *M. bovis* LTA and ELISA negative.

On 11 July 1994, shortly before slaughter, the CCT produced increases in skin thickness up to 22 mm at the bovine tuberculin injection sites in Hinds 1, 4 and 5, a 3 mm thickening in Hinds 3 and 6, and a 1 mm increase in skin thickness at the site of bovine tuberculin injection in Hind 2. Avian tuberculin injection sites had an increase in thickness of up to 1 mm. Hind 2 was thus the only one negative to the CCT.

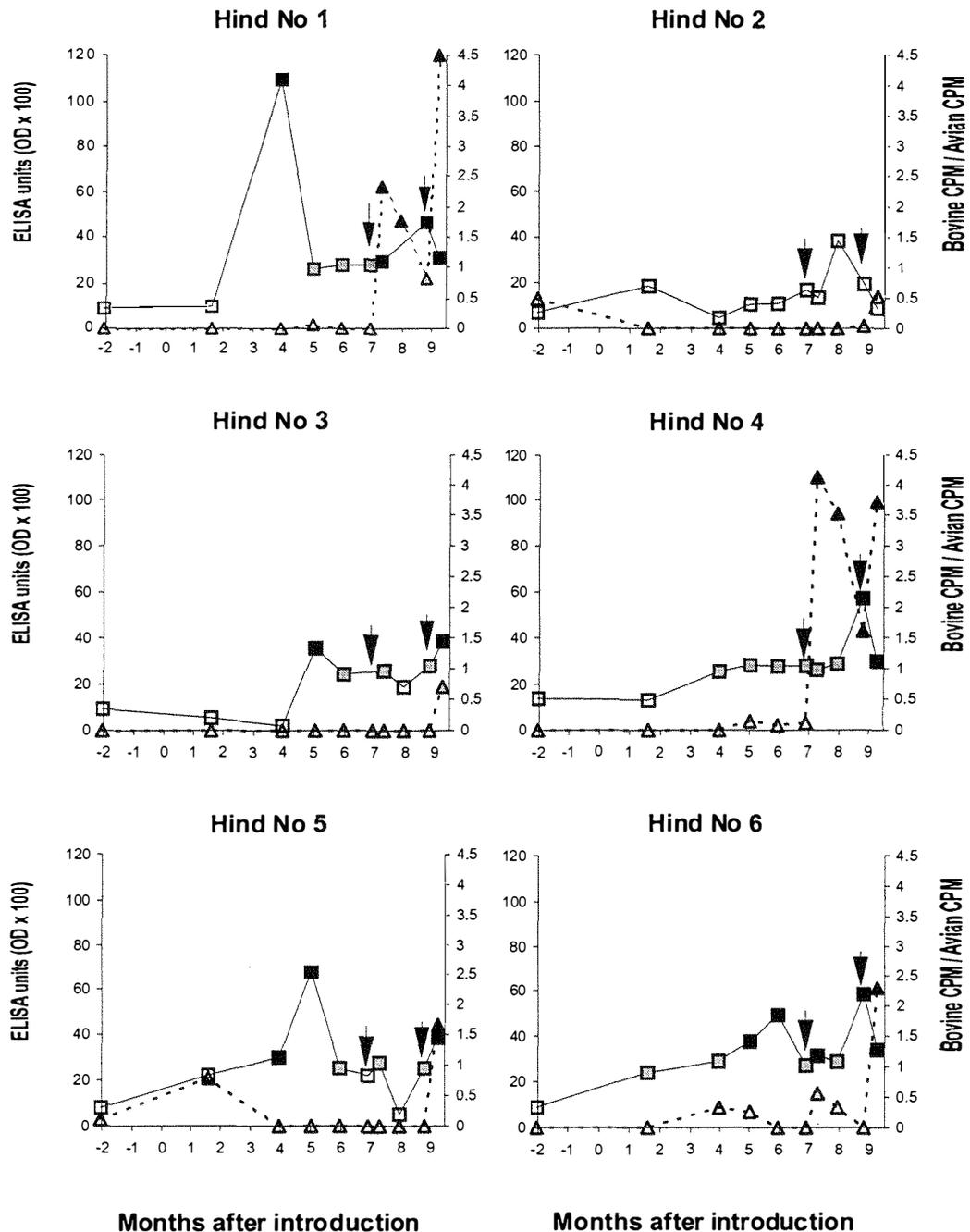


Figure 5-2. Bovine serological ELISA (- - Δ - -) and LTA (— \square —) responses to bovine PPD for hinds in Group 1. The open data points represent negative test interpretation, the shaded points equivocal results, and the solid points positive results, when each is interpreted in isolation. The arrows show the timing of the tuberculin injections

Cultures and histopathology

Mycobacterium bovis was recovered off tonsillar swabs twice from Hind 6 whilst alive, and on all three sampling occasions from Hind 1. Detailed necropsies of Group 1 animals revealed a gross lesion typical of tuberculosis in Hind 3 only. A single caseous lung tubercle 4 mm in diameter was found. Single caseous tonsillar

crypt lesions 2-3 mm in diameter were seen on the right and left sides in Hinds 1 and 2, and on the right side only in Hind 6, but these were not typical of bovine tuberculosis lesions found in other lymphoid tissues, but were typical of normal ruminant tonsillar crypt pathology caused by a range of organisms (Payne and Derbyshire, 1963). A single mucoid crypt lesion was seen in Hind 4. These tonsillar lesions and surrounding tissue formed part of the sample submitted for culture. Tissues submitted for histopathology included sections of liver with white parasitic foci, various lymph nodes with cream-coloured areas of parenchymatous calcospherulosis, small lymphoid hyperplastic lung nodules and the one tuberculous lung lesion. Sections of tonsils (not including portions of the macroscopic lesions) from four animals showed non-specific suppurative crypt lesions often centred around vegetable debris or club forming bacterial colonies, and granulomatous inflammation about calcospherules. Histopathology typical of tuberculosis, with acid-fast bacilli, was present only in the lung tubercle in Hind 3.

Cultures confirmed that the five deer returning positive blood tests and CCT results were all infected (see Table 5-II). The lung nodule in Hind 3 contained *M. bovis*, as did the oropharyngeal tonsils of all four deer without typical lesions, and the medial retropharyngeal lymph nodes in Hinds 4 and 6. Of the six REA types of *M. bovis* that were recovered from possums throughout the five years of the longitudinal study, Hinds 3 and 5 were infected with restriction type 10b and the remaining three infections were type 4. Hind 2 was culture-negative.

Social rank and sequence of infection

The concurrent observational studies on Group 1 deer utilising sedated non-tuberculous possums (Sauter, 1995), revealed that Hinds 1 and 5 were the most dominant and inquisitive (Table 5-II) and most likely to have physical contact with possums. These were the first to show positive *M. bovis* LTA responses. Conversely Hind 2, which remained blood and skin test negative and which was both lesion and culture negative, had the lowest social rank in the group. This hind was very timid, and was the least likely to approach a possum. Hind 3, which was the last to show bovine reactivity in the BTB, was ranked fifth.

Table 5-II. Summary of social ranking, test and culture results of Group 1 deer

Hind.	Social rank ^a	Date of initial LTA reactivity		Bovine ELISA after PPD ^b 15/5/94	CCT result ^b 11/7/94	Gross lesion status ^b 26/7/94	Culture status ^b	Isolation sites ^c	REA type
		Equivocal	Bovine						
1	1		16/2/94	+	+	-	+	T	4
2	6			-	-	-	-		
3	5		20/3/94	-	+	+	+	Lung	10b
4	4	16/2/94	11/7/94	+	+	-	+	T, MR	4
5	2		16/2/94	-	+	-	+	T	10b
6	3	7/12/93	20/3/94	±	+	-	+	T, MR	4

^a From Sauter (1995).

^b + represents a positive result, - represents a negative result, ± is equivocal.

^c T indicates an infected oropharyngeal tonsil and MR the medial retropharyngeal lymph node.

Group 2

At the BTB test before introduction to the study site, all six hinds showed *M. avium* reactivity. Four of the six showed positive *M. bovis* LTA responses, one showed *M. avium* reactivity and the sixth (Hind 9) produced a “no data” result 2 months after introduction. Subsequent BTB tests generally produced avian reactivity, but one hind showed an equivocal bovine response on 17 October 1994, and Hind 9 again furnished a “no data” result.

No swabs from this group cultured *M. bovis*. Hind 9 died on 18 October 1994, 1 day after sampling, from what appeared to be an acute clostridial wound infection, and Hind 11 died on 8 December 1994 from an acute septicaemic condition, possibly malignant catarrhal fever. Detailed necropsies performed on the deer, and culture of tissues as above, provided no evidence of *M. bovis* infection.

None of the Group 2 deer showed any interest in, or interacted closely with sedated possums (Sauter, 1995). In April 1995, the remaining four hinds escaped from the enclosure. Up until that time, and including a test performed on the day before escape, these animals showed no further *M. bovis* BTB reactivity.

Domestic cattle

Three skin test reactor cattle were removed from adjacent to the deer enclosure during the period in which the hinds became infected. At necropsy, the first two removed on 10 December 1993, were both found to have 15 mm lung tubercles and associated lesions in the draining tracheobronchial lymph nodes. *Mycobacterium bovis* REA type 4 was isolated from these lesions. The third, removed on 23 March 1994, had tubercles in one medial retropharyngeal lymph node, from which *M. bovis* REA type 10b was isolated. Culture of the oropharyngeal tonsils of this animal failed to isolate *M. bovis*.

Discussion

This study has demonstrated for the first time the natural infection of deer with *M. bovis* from tuberculous possums and provided evidence for the mode of transmission. It is also the first published longitudinal study of the immunological response of farmed red deer following natural infection. Other studies have followed the response to artificial infection (Carter *et al.*, 1984; Corrin *et al.*, 1987). Data supports the hypothesis that the principal mode of transmission from possums to deer is via the oral route, involving the oropharyngeal tonsil, after inquisitive deer investigate moribund tuberculous possums, as described by Sauter and Morris (1995a). Infection of the oropharyngeal tonsil is unlikely to have arisen as a consequence of entry of bacilli via the nasal cavity. Particulate material alighting on the nasal mucosa is passed caudally by ciliary action over the nasopharyngeal tonsil prior to swallowing directly from the nasopharynx. The nasopharyngeal tonsil possesses the same immunologic function as the oropharyngeal tonsil, and is the only structure in the nasal cavity capable of taking up macromolecular antigens (Chen *et al.*, 1989). Other studies involving 56 tuberculous deer have found that the nasopharyngeal tonsil was infected in 4 cases (2 with concurrent oropharyngeal tonsillar infection), whereas the oropharyngeal tonsil was found infected in 34 deer (Chapter 6). This suggests that inhalation of *M. bovis* into the nasal cavity with subsequent establishment of local infection is less common than orally acquired infection. The observations in this study are consistent with the finding that artificial infection via the tonsillar route closely mimics naturally acquired tuberculosis (Mackintosh *et al.*, 1993). The belief that natural deer to deer

transmission, capable of establishing infection and producing disease, may be less important than infection from wildlife reservoirs in some situations (Mackintosh and Griffin, 1994), especially early in the course of the disease when the excretion of bacilli is minimal, is also supported by these results. However, while this evidence supports the hypothesis of possum to deer transmission via the oral route, deer to deer transmission amongst Group 1 cannot be completely discounted.

Discovery of tuberculous possums on the study site coincided with the expected summer peak in possum disease prevalence (Jackson, 1995), but the number found near the deer paddocks was unexpectedly high. During the first 3 months, Group 1 hinds could have been exposed to six known tuberculous possums. Five of these six hinds probably became infected through close interactions with at least two tuberculous possums, although no such interaction was actually seen. This exposure is likely to have occurred prior to mid January for Hinds 1, 4, 5 and 6, which showed early *M. bovis* LTA reactivity. Hinds 3 and 5 were probably infected by the possum with the draining lesion which denned in the deer enclosure, as they and the possum shared the same restriction type (10b). The possum(s) which may have been the source of infection for the other three hinds would have been amongst the five with type 4 found during the first 3 months. An alternative explanation for the infections of the deer may have been contact with other infected wildlife or domestic cattle in the area. However, this is unlikely as there were very few wild deer or pigs in the immediate vicinity and sign was rarely seen during the period when the hinds became infected. Concurrent, and later predator trapping around the study site suggested that other wildlife hosts of *M. bovis* were not abundant, either on the farm nor in the vicinity of the deer paddocks. One *M. bovis* REA type 4 infected hedgehog was trapped near the deer enclosures in October 1994 after the Group 1 hinds had been removed. It was believed that this animal acquired the infection from the carcasses of tuberculous possums previously dying in the area. Despite the prevalence of tuberculosis in ferrets in the locality being very high (Chapter 4), the low abundance of this species, and a lower level of interest in ferrets demonstrated by deer (Sauter and Morris, 1995a), would make infectious contact with the hinds unlikely. The cattle adjacent to the deer enclosure were also thought to have become infected through contact with the tuberculous possums. Although there was the potential for fence contact between the cattle and deer, close

interactions between cattle and deer are not common, and there are few reports in the literature of suspected cattle to deer transmission of tuberculosis. Neither the deer nor the cattle investigated in this study were likely to have been sufficiently infectious, to readily infect and establish disease in the other species.

The more dominant and inquisitive deer were the first to become infected, which was consistent with the theory proposed by Sauter and Morris (1995b) for both deer and cattle. Hind 3, apparently the last infected, had the second to lowest rank and most timid nature of the infected deer. This hind's lung tubercle possibly arose through aerosol transmission while cautiously sniffing a tuberculous possum. Infection via direct contact, although a possibility, is not likely to produce a lung tubercle without leaving evidence of infection at other sites (Chapter 6). It is possible that Hind 2 may have been innately resistant to *M. bovis* infection, and failed to show any *M. bovis* LTA reactivity following infection. However, that hind was the most timid, and probably remained uninfected as she was the least likely to approach a possum closely. Observational studies in cattle have also shown that they will place themselves at risk of acquiring tuberculosis through closely investigating simulated moribund tuberculous possums (Paterson, 1995) and that the risk of infection is directly related to social rank (Sauter and Morris, 1995b).

All five deer with *M. bovis* isolates were positive to both the CCT and the BTB. They were not positive to the BTB on every occasion, however, despite the likelihood that they were continuously infected from the time of the first positive LTA (or the second equivocal test, in the case of Hind 4). The chronological relationship between the presence of tuberculous possums, and the LTA responses of the deer infected with the same type, suggest the reactions took between 1 and 2 months to develop. This is in accord with the observations of Mackintosh *et al.* (1993) who have shown that LTA responses to bovine tuberculin usually occur 4-6 weeks after experimental infection.

Positive LTAs in deer, which had no gross lesions at necropsy, have been reported elsewhere (Griffin *et al.*, 1991). Such deer may have inapparent tonsillar infections as this is a common site of colonisation by *M. bovis* in deer, as observed in this study and elsewhere (Chapter 6). These observations suggest that the cellular immune response may be protective in some circumstances, as proposed by Griffin *et al.* (1993a). Further, it could be suggested that the infecting dose was small, and

that the deer, which maintained good condition, were not suffering undue stress which could reduce host resistance to disease. Susceptible deer may develop severe tuberculosis after 4 months when inoculated experimentally with as few as 10 organisms into the tonsil (Mackintosh and Griffin, 1994).

Despite negative cultures from most of the tonsillar, nasal, tracheal and faecal samples, infected hinds could have excreted low numbers of *M. bovis*, as the sampling occurred infrequently, and the small sample of material from the swabs, severe decontamination procedures for faeces, and freezing, will all contribute to reduce the sensitivity of *M. bovis* isolation techniques. The finding of *M. bovis* in the tonsillar fossae indicates that there must have been shedding of bacilli from the lymphoepithelium of at least some of the infected oropharyngeal tonsils. The saliva of these infected animals could have been infectious.

The transient *M. bovis* LTA reactivity of the second group of deer may have resulted from infection by interactions with the infected Group 1 hinds. Shortly after introduction, all deer were seen to rub noses through the separating fence and after settling in, all hinds were run together. Group 2 hinds were never interested in investigating simulated moribund tuberculous possums provided to them (Sauter, 1995). Pasture contamination is less likely to have been involved, as survival of *M. bovis* cultures absorbed on cotton ribbon placed on herbage in this area was found to be only a matter of days (Jackson *et al.*, 1995c) and the few bacilli consumed will be diluted by other ingesta, making contact with the tonsils improbable. Contamination of water troughs would also be similarly diluted. If feed or water were a source of *M. bovis*, it is probable that Hind 2 in Group 1 should also have shown evidence of infection. The transient *M. bovis* LTA reactivity of Group 2 hinds may indicate exposure without establishment of infection, possibly followed by immunity, as proposed by Griffin *et al.* (1993a). Thus few conclusions can be drawn from Group 2 other than that their behaviour towards possums was different from Group 1, and that this behavioural difference supports the hypothesis that inquisitive deer are more at risk from infection from tuberculous possums than uninquisitive deer. It must be noted, however, that tuberculous possums were found only for a short period after the introduction of Group 2, thus limiting the possible period of exposure.

Infection of the oropharyngeal tonsils in deer often occurs without lesion development, or with lesions indistinguishable from the normal crypt pathology of these organs (Chapter 6). In the past this has led to the tonsillar tissues being overlooked as sites of infection during necropsies, and has decreased the sensitivity of diagnosis by either gross necropsy, or mycobacterial culture. It is recommended that the oropharyngeal tonsils should be examined and cultured in all deer suspected of being tuberculous.

This study has, for the first time, followed the course of a natural bovine tuberculosis infection in deer. Observations indicated that direct transmission of infection from possums was probably involved, and have helped establish links between deer social structure and behaviour, and the risk of infection. This study has also provided further evidence for the importance of the oropharyngeal tonsil in the pathogenesis of infection.

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The PhD candidate was responsible for the overall management of, and collection and dispatch of samples from, the deer at the study site. Field assistance was provided by Carola Sauter, and on occasion by Peter Wilson. The candidate was also responsible for the interpretation of results and preparation of the manuscript.

CHAPTER 6

BOVINE TUBERCULOSIS IN WILD RED DEER

Abstract

Free-ranging red deer (*Cervus elaphus*), 30 from the Castlepoint area and another 76 from the Hauhungaroa Range, both tuberculosis endemic areas, were examined for the presence of *Mycobacterium bovis* infection. Examination of these wild deer was supplemented by further samples gathered from 46 farmed deer killed at two Deer Slaughter Premises. Necropsies were performed on whole deer or parts thereof, and a standard set of tissues and excretion site samples were collected for mycobacteriology. These samples included: a sub-sample of both the oropharyngeal tonsils; the nasopharyngeal tonsil; a sub-sample of both medial retropharyngeal lymph nodes, and a combined sample of the caudal mediastinal, left tracheobronchial and the cranial tracheobronchial lymph nodes; faeces; urine, and swabs of the nasal cavity, oropharynx and tracheal mucosa.

Fifty eight infected deer were identified, with the prevalence of bacteriologically confirmed infection in the wild deer being 32%. In no studies in wild deer in any other country, have prevalence estimates approached this figure. In New Zealand a comparable prevalence of tuberculosis has previously been reported only from wild deer originating in areas with endemic tuberculosis in possums (*Trichosurus vulpecula*). There is now strong evidence to suggest that a high prevalence of tuberculosis infection in wild deer can only be maintained through association with infected possums. Amongst the wild deer there was a significant trend for the prevalence to increase with increasing age. Only one of 18 deer less than 1 year-old was infected and mature deer were 12 times as likely to be infected as those under 1 year of age. Infected older deer were also less likely to show typical gross lesions than younger animals. *Mycobacterium bovis* was isolated from oropharyngeal tonsil of 61% of the infected deer, and was the most frequently infected site. Approximately half of the infected oropharyngeal tonsils showed no gross lesions, but where tonsillar crypt lesions were identified, they were twice as likely to be found in infected deer as those uninfected. This suggests that only a proportion of crypt lesions could have been attributable to *M. bovis* infection. Infection in the oropharyngeal tonsil was strongly associated with the presence of *M. bovis* in the draining medial retropharyngeal lymph nodes. The nasopharyngeal tonsil was found to be infected in four (7.5%) animals. This is the first time infection of the nasopharyngeal tonsil has been reported in any species, and it is postulated that this is due to infection by large airborne particles carrying bacilli. There was a

suggestion from the data that infected male deer were two to three times as likely to have gross lesions as infected females, and that the disease in males was more severe. It is proposed that this may have been associated with stressors operating during the sexually active period of males. *Mycobacterium bovis* was recovered from 4 of 53 (7.5%) oropharyngeal swabs, 1 of 53 (1.9%) tracheal and nasal swabs, from 1 of 46 (2.2%) faecal samples and from no urine specimens. This suggests that significant bacillary excretion from infected deer was uncommon, and that the few animals which are severely affected, may be the only highly infectious deer.

This study has confirmed the importance of lymphoepithelial tissues as primary sites for the establishment of *M. bovis* infection, and for subsequent excretion of organisms. These sites, such as the oropharyngeal tonsil and nasopharyngeal tonsil usually remain free of gross lesions when infected, but if gross lesions develop they are impossible to differentiate macroscopically from lesions due to other causes. These results also support the existence of an association between the presence of infected possums and a high prevalence of disease in free-ranging red deer populations in the same area. Furthermore, the results also suggest that if tuberculosis is eradicated from possum populations, that deer may still be able to maintain the disease amongst themselves, but at a low prevalence.

Introduction

Red deer were first introduced to New Zealand in 1851 (Livingstone, 1994). Wild populations are now widespread over most of the South Island, and much of the central and southern North Island. There are currently estimated to be 250,000 wild deer in New Zealand with the majority of these being red deer (*Cervus elaphus*) which are present in most environments in which deer are found. As a result of hunting pressure, deer populations are largely confined to sheltered forested country, where they share the ecosystem with a variety of other potential hosts of tuberculosis, notably the brushtail possum (*Trichosurus vulpecula*).

Tuberculosis was first noted in wild red deer on the West Coast of the South Island in 1956 (Livingstone, 1994), and since then *M. bovis* has been isolated from numerous wild deer (De Lisle and Havill, 1985; Nugent, 1994; Nugent and Proffitt, 1994). The disease is widespread in wild deer populations, and has been seen mostly in animals from the Wairarapa, Central North Island endemic area and the West Coast, but a few have also come from Fiordland, Otago and Southland (Mackintosh and Beatson, 1985). It is noteworthy that areas such as Taranaki and Northland, which do not have significant wild deer populations, also do not have tuberculous possum populations.

It has been speculated that the wild deer of New Zealand may have been infected for a long period, and were responsible for the initial introduction and establishment of tuberculosis in possum populations in what are now regarded as endemic areas (Morris and Pfeiffer, 1995). This hypothesis has been given recent support by a number of instances where farmed deer appear to have been responsible for the introduction of tuberculosis to possum populations (Mackereth, 1993; de Lisle *et al.*, 1995). The determination of whether wild deer are likely to maintain infection in their own populations independently of outside sources, will be important to tuberculosis control efforts of the future, particularly if the eradication of the disease from possum populations becomes feasible.

Suppurating sinuses, a common feature of the disease in deer, are probably responsible for the bulk of deer to deer transmission observed in farmed deer. Deer with these lesions have been present in numerous severe outbreaks of tuberculosis (Beatson *et al.*, 1984; Robinson *et al.*, 1989; Atkinson, 1993; Mackintosh and Griffin 1994; Whiting and Tessaro, 1994), but have been absent where disease

transmission has been low (Paterson 1993; Mackintosh and Griffin 1994; Mackintosh *et al.*, 1995). The preponderance of lesions in the medial retropharyngeal lymph nodes (Hathaway 1994), suggests that most bacillary uptake occurs from the oral cavity. Experimental inoculation of the tonsillar crypts has been shown to reliably reproduce a disease state similar to that seen naturally, thus supporting the importance of the oral route of infection (Mackintosh *et al.*, 1993; Mackintosh *et al.*, 1995).

The majority of infected farmed deer in New Zealand have single lesions containing few acid-fast bacilli (AFB) (Hathaway *et al.*, 1994), suggesting that most have a degree of resistance to the disease, and that they are incapable of shedding large numbers of bacilli. A proportion of deer may also be slaughtered during the early stage of disease, when lesion and bacillary numbers are low. The transmission of disease between deer is typically slow (Mackintosh and Griffin, 1994; Mackintosh *et al.*, 1995) unless the animals are subjected to severe stress (Kollias *et al.*, 1982; Robinson *et al.*, 1989) and consequently show heightened adrenal glucocorticoid responses (Thomson *et al.*, 1994). In genetically susceptible animals this allows disease progression (Griffin *et al.*, 1993b; Mackintosh *et al.*, 1995), and bacillary excretion to escalate.

New Zealand, British and North American data suggests that the disease typically exists at low prevalence in free-ranging deer populations for prolonged periods in the absence of other sources of infection (Philip, 1989; Rose 1987; Anon., 1990; Fletcher, 1991 and 1990; Schmitt, 1995; Nugent and Mackereth, 1996; B. Corso, pers. comm.). In Britain and Ireland, where infected badgers are found, the prevalence of tuberculosis in deer is higher, but still apparently between 1 and 5%, which suggests that there is some transmission from badgers (*Meles meles*) to deer, as well as to cattle (Dodd, 1984; MAFF reports, 1985 and 1986; Philip, 1989). In New Zealand the 20 to 30% prevalence found in wild deer in the central North Island endemic area has been argued to support the involvement of tuberculous possums in maintaining the high prevalence of disease (Nugent, 1994; Nugent and Proffitt, 1994). This suggestion is supported by the intimate interactions of deer with sedated possums (Sauter and Morris, 1995a), and by the slow spread of tuberculosis in the area east of Turangi. Here the disease seems to have remained

confined to an area known to contain tuberculous possums, despite the potential for it to have spread widely with deer dispersal (Nugent and Proffitt, 1994).

The aims of this study were to investigate the epidemiology of the disease in free-ranging populations, and to acquire a better understanding of the pathogenesis of tuberculosis in deer. This project also investigated the likely routes of transmission between deer, and attempted to determine whether the requirements for deer to be considered as reservoir hosts of tuberculosis were fulfilled, under New Zealand conditions.

Materials and Methods

Origin of deer

Whole deer or parts thereof were gathered from five sources (Table 6-I). The majority of the wild deer were recovered by helicopter shooting over the Hauhungaroa Range, to the west of Lake Taupo, in the Central North Island endemic area. Other wild deer were shot by ground hunters from around the Castlepoint district in the Wairarapa endemic area. Both of these are areas in which tuberculous possums are regularly found. However, the high cost and practical difficulties of obtaining deer by these methods, meant that the total number of infected deer in these samples was limited. To supplement the data from the wild deer, and to improve power in analyses intended to gain a better understanding of the pathogenesis of tuberculosis, samples were also collected from skin test positive farmed deer killed at Deer Slaughter Premises (DSPs). This specimen collection was carried out when there was a history suggesting that the animals being slaughtered were likely to be infected with *M. bovis*. Data from the necropsy examination of the farmed deer used in the natural tuberculosis transmission trial (Chapter 5) were also used in the analyses.

Data collection

Data collected on each deer at the time of necropsy included: Date; area of origin; whether wild or farmed; sex; estimated age; portions of carcass available for examination; presence and type of tonsillar crypt lesions; whether fibrous tags or adhesions were present on the pleura; presence of small (up to 8mm) grey homogeneous pulmonary nodules; presence of lungworm (*Dictyocaulus viviparus*) in the major bronchi; description of lesions; provisional diagnosis; and samples kept for mycobacteriology or histopathology.

Ageing

The majority of the wild deer had their age determined by teeth eruption and examination of cementum annuli using the technique described by Fraser and Sweetapple (1993). The ages of the remaining wild deer were estimated from the body size and antler growth, based on the assumption that deer were born in December. For analytical purposes the ages were recorded both in years, and also by four age classes (Table 6-III).

Table 6-I. Description of the deer examined and prevalence of *M. bovis* infection, including area of origin and portions examined

Origin and date	Type ^b	Parts examined ^a					Total deer	Number infected	Prevalence (95% CI)
		HTAB	HTA	HTB	HT	H			
Hauhungaroas 1994-1995 ^c	W	40	26	4	6		76	25	0.33 (0.22 - 0.44)
Castlepoint ^d 1993-1995	W		7		21	2	30	9	0.30 (0.14 - 0.46)
Deer trial July 1994 ^e	F	7	2		1		10	5	0.50 (0.19 - 0.81)
Feilding DSP Feb-March 1995	F	24					24	0	0
Mamaku DSP March 1995	F	22					22	19	0.86 (0.72 - 1.0)

a HTAB = head, thoracic contents, abdominal contents and body; HTA = head, thoracic contents and abdominal contents; HTB = head, thoracic contents and body; HT = head and thoracic contents; H = head only

b W = wild; F = farmed - skin test positive

c 52 deer recovered between March and June 1994, and 24 between October and December 1995

d deer recovered between November 1993 and December 1995

e animals from the Castlepoint trial reported in Chapter 5, including two that were shot following escape from the trial site

Sample collection

A protocol describing the full post-mortem examination of deer for tuberculosis infection is included in appendix I. Tissues were handled in a similar manner for all deer, but examination procedures varied, depending upon circumstances prevailing at the time. These variations depended upon the parts of carcasses presented, and gun shot damage which made some tissues unavailable for collection or inspection. The carcasses of the first group of 44 of the 76 deer from the Hauhungaroas, which appeared to be free of tuberculosis, were also sold to a Game Packing House (GPH), which meant that body nodes from 32 deer were unavailable for examination. The majority of samples from wild deer around Castlepoint were frozen and thawed prior to inspection, whereas deer from other areas were examined when fresh.

Swabs containing transport medium (Transwab, Medical Wire and Equipment Co. Ltd) were used to gather material from the mucosal surface, up to a distance of 10 cm inside the nasal cavity caudal to the nares; the mid-cervical section of the transected trachea; and the oropharynx. The pharyngeal sample was taken from the area surrounding the oropharyngeal tonsils, but the tonsillar fossae were deliberately avoided. A faecal sample comprising several pellets was extracted from the cut end of the rectum, and a urine sample was removed from the bladder following needle puncture. All major lymph nodes were laid out on pre-labelled paper sheets and sliced at 2 mm intervals. Lungs initially had the major bronchi opened and examined for the presence of lungworm, and were then sliced to a 2 cm thickness to facilitate thorough palpation and visual examination. Four tissue pools of approximately 5 to 8 g each were retained from each deer for mycobacterial culture. These comprised a sub-sample of both the oropharyngeal (palatine) tonsils (including any visible lesions), the nasopharyngeal tonsil (adenoid), a sub-sample of both medial retropharyngeal lymph nodes, and a combined sample of the caudal mediastinal, left tracheobronchial and the cranial tracheobronchial (apical) lymph nodes. Other suspicious lesions were kept for culture, (and occasionally for histopathology), if typical lesions were not included in the other tissue pools already retained. To avoid cross-contamination of samples for culture, instruments were immersed in boiling water as appropriate. Isolation of *M. bovis* from faeces, urine, and the nasal, tonsillar and tracheal swabs was attempted if cultures from the submitted tissue pools confirmed the animal as tuberculous.

Sample examination

Tissues removed for bacteriology were stored in sterile plastic containers, and together with swabs and other samples, stored at -84°C. Samples were later submitted to the AgResearch tuberculosis laboratory, Wallaceville, for culture. Routine culture techniques described by Buddle *et al.* (1994) were used to isolate *M. bovis* from tissue samples. Urine and faeces were cultured using the method described by Jackson *et al.* (1995b). Swabs were cultured by vortexing them in 10 ml of sterile-distilled water and then 5 ml of this solution was decontaminated by the addition of an equal volume of 0.75% w/v of cetyl pyridinium chloride. The remainder of the culture procedure for the swabs was the same as that used for tissues. Animals or tissues from which *M. bovis* were isolated were termed

‘infected’, and animals or tissues showing tuberculous lesions were termed ‘diseased’.

From selected cases, formol-saline fixed tissues were held for later examination. Paraffin-blocked tissue prepared from this material by routine histological processing was sectioned and stained using haematoxylin/eosin and Ziehl-Neelsen methods.

Statistical analyses

Unconditional logistic regression, employing backwards stepwise elimination where necessary, was used to examine the relationship between a range of independent covariates and dichotomous dependent variables (Table 6-II). Univariate screening was initially used to identify significant covariates, and those with p-values less than 0.5 were included in the regression analyses.

Table 6-II. Summary of variables used for logistic regression analyses. Highlighted covariates were removed by preliminary univariate screening

Dependent variable	N	Covariates		Results table
		Categorical	Continuous	
Infection status of wild deer	106	sex, age (3 levels) ^a , sex*age		Table 6-VI
Presence of tonsillar crypt lesions	153	sex, infection status, sex*infection status	age, sex*age	Table 6-VIII
Presence of grey pulmonary nodules	90	sex, infection status, lungworm presence, sex*infection, sex*lungworm	age	Table 6-IX
Presence of typical tuberculous lesions	58	sex	age, sex*age	Table 6-VII
Presence of small fibrous pulmonary tags	75	sex, infection status, lungworm, grey pulmonary nodules	age	

a age was incorporated as a categorical variable, with three classes, i.e. weaners, yearlings and mature animals (mature and aged classes combined), after examination by the χ^2 test for trend established that prevalence of disease did not increase linearly with age.

Logistic regression analyses were conducted with the aid of the statistical software package SPSS version 7 (SPSS Inc., Chicago, IL). All contingency table analyses were performed within the program Epi Info version 6.02 (Centers for Disease Control and Prevention, Atlanta, GA.). Where appropriate, 95% confidence limits have been presented with results.

Results

Description of the deer

Between November 1993 and February 1996, 162 red deer were examined (Table 6-I). The completeness of the examinations is shown by the number of complete carcasses or portions examined. The whole carcass was examined in less than half of the wild deer.

The class of deer, sex and age distribution are presented in Table 6-III. Ninety-seven wild deer were aged by examination of teeth eruption status and cementum annuli. The youngest deer was estimated to be 4 months of age, and the oldest deer 10.3 years of age. Eight mature farmed deer for which the age was not known were arbitrarily given an age of 2.65 years, which was the average age for deer in this category.

Table 6-III. Class, age and sex distribution of deer examined

Age	Farmed			Wild		
	female	male	total	female	male	total
Weaners ≤12 m			0	6	12	18
Yearlings 13 - 23 m	13	16	29	6	23	29
Mature 24 - 48 m	13	11	24	11	30	41
Aged ≥49 m	3	0	3	12	6	18
Total	29	27	56	35	71	106

Infection status and relationships

Mycobacterium bovis was isolated from 58 (35.8%) of the deer examined. *Mycobacterium avium* was isolated from a respiratory lymph node pool from one farmed deer without gross lesions. The prevalence of infection (culture positive) in the various groups of deer is presented in Table 6-I. The combined prevalence for all wild deer from the two locations was 0.32 (0.23 - 0.41).

Table 6-IV. Summary of gross and bacteriological findings in 58 infected deer

Site	No. examined	No. typical gross lesions ^a	Proportion with typical gross lesions	No. cultured	No. culture positive	Proportion culture positive	Proportion lesion -ve /culture +ve
OP Tonsil	56	1	0.018	56	34	0.607	0.971
NP Tonsil	53	0	0	53	4	0.075	1.00
Medial retro-pharyngeal Inn.	55	12	0.218	55	21	0.382	0.429
Head-associated ^b	58	13	0.224	58	39	0.672	0.666
Respiratory Inn.	58	17	0.310	46	22	0.478	
Lung ^c	58	15	0.259	7	4	0.571	
Total thoracic	58	20	0.345	52	25	0.481	
Mesenteric Inn.	47	14	0.298	5	4	0.800	
Peripheral Inn.	52	4	0.077	7	2	0.286	

a characterised by purulent/caseous appearance at the centre of lesions, which can be grossly differentiated from most other pathological conditions

b includes all head-associated lymph nodes and tonsillar sites

c does not include pleuritis or grey pulmonary nodules

Age associations

There was a significant trend for increasing prevalence of infection as the wild deer became older ($p = 0.005$). The age prevalence of infection is summarised in Table 6-V. There was little difference in prevalence between the mature and aged classes, suggesting that the trend was non-linear.

Table 6-V. Chi² analysis for trend examining the relationship between age class and infection status in 106 wild red deer

Age class	Number without infection	Number with infection	Prevalence	Odds ratio
Weaners	17	1	0.056	1.0
Yearlings	21	8	0.276	6.5
Mature	24	17	0.415	12.0
Aged	10	8	0.444	13.6

$$\chi^2_3 = 7.92, p = 0.005$$

Multivariate analysis of sex and age effects on the prevalence of disease is presented in Table 6-VI. Neither sex, nor the interaction term of sex and age had any significant effect on the probability of infection. Age was the only influential independent variable, with the odds of infection in yearling deer 6.5 times as great as weaners, and the odds of infection in mature animals 12.5 times as great as weaners.

Table 6-VI. Final logistic regression model examining the effect of age class on the infection status of 106 wild red deer. Weaner age class is used as the standard

Variable	Coefficient	SE	DF	P	Odds ratio(95%CI)
Intercept	-2.83	1.03	1	0.006	
Age			2	0.035	
Yearling	1.87	1.11	1	0.092	6.5 (0.74 - 56.9)
Mature	2.52	1.06	1	0.017	12.5 (1.6 - 100.1)

Deviance = 122.3, $\chi^2_2 = 10.7$, $p = 0.005$

The presence of typical gross lesions in infected deer was found to be significantly associated with the age of the deer (Table 6-VII), the prevalence of such lesions decreasing as the age increased. Although there was not a significant statistical association between sex and the presence of typical lesions, there was an indication that infected males were two to three times as likely as females to be found with lesions (univariate $p = 0.18$). The four deer judged to have the most severe gross lesions, either because of size and/or number of sites involved, were all males with estimated ages of 1.3 (farmed deer), 3.3, 3.83, and 4.4 (wild deer) years old.

Table 6-VII. Final logistic regression model examining the effect of age on the prevalence of typical gross lesions in 58 infected deer

Variable	Coefficient	SE	P	Odds ratio (95% CI)
Intercept	2.27	0.65	<0.001	
Age	-0.48	0.21	0.019	0.62 (0.41 - 0.92)

Deviance = 62.0, $\chi^2_1 = 6.28$, $p = 0.012$

Sites of infection

Oropharyngeal tonsils

Tonsillar crypt lesions usually ranged in appearance from small foci of translucent mucopurulent liquid through to firm yellow caseous deposits in the crypt lumen (Figures 6-1 and 6-2), although occasional white granular lesions were seen. Crypt debris and exudate typically caused little crypt distension and it was uncommon for a lesion to be over 3 mm in diameter. Vegetable matter, including grass seeds were commonly found in the crypts, often encrusted in a yellow caseous coating. Even though the aetiology of these lesions containing vegetable matter seemed apparent, they were recorded no differently to other crypt lesions. Two deer had a purulent

tonsillitis with a typical tuberculous appearance, both with tonsillar lesions over 1 cm in diameter. Only one of these purulent lesions was tuberculous and this came from a cachectic wild deer (2730) with very advanced terminal tuberculosis and numerous large lesions. The other purulent tonsillar lesion came from an uninfected deer, and was the only gross lesion visible in the portions of the carcass examined. From the gross descriptions recorded at necropsy it was not possible to ascribe any particular tonsillar lesion type to the possible presence of *M. bovis* infection in the tonsil, and indeed 16 of 34 deer with oropharyngeal tonsillar *M. bovis* isolates showed no gross lesions in the oropharyngeal tonsil whatsoever.

Seven deer had oropharyngeal tonsillar tissues examined histologically (four described in previous chapter). Significant lesions were found in Deer 2730 only. These tonsils showed a granulomatous tonsillitis in which numerous giant cells, some containing AFB, were present.

Analysis of the factors associated with the presence of tonsillar crypt lesions is presented in Table 6-VIII. The only covariate shown to have a significant association was the infection status, with infected animals being twice as likely as *M. bovis*-free animals to have lesions found in the crypts. However, contingency table analysis of the relationship between infection of the oropharyngeal tonsil and lesion presence in the tonsillar crypts was suggestive of, but failed to show a significant relationship between the two observations ($\chi^2_1 = 2.22$, $p = 0.14$; odds ratio $0.78 < 1.79 < 4.12$). Analysis of the relationship between infection of the oropharyngeal tonsil or the medial retropharyngeal lymph nodes, and the presence of tonsillar crypt lesions did show a significant relationship ($\chi^2_1 = 4.47$, $p = 0.035$, odds ratio $0.99 < 2.23 < 5.06$). Taken together these observations suggest that there is a significant relationship between infection in the oropharyngeal tonsil and the presence of crypt lesions, but that some cultures of infected oropharyngeal tonsils may have failed to isolate the organism.

Table 6-VIII. Final logistic regression model examining the effect of infection status on the presence of tonsillar crypt lesions (n = 153)

Variable	Coefficient	SE	P	Odds ratio (95% CI)
Intercept	-0.59	0.21	0.005	
Infection	0.70	0.34	0.042	2.01 (1.03 - 3.93)

Deviance = 203.8, $\chi^2_1 = 4.17$, $p = 0.04$

Nasopharyngeal tonsil

There were four deer in which *M. bovis* was isolated from the nasopharyngeal tonsil. None showed gross lesions, and one was obtained from Deer 2730, from which isolates of *M. bovis* were obtained from multiple sites, including the nasal cavity.

Medial retropharyngeal lymph nodes

Of the 21 medial retropharyngeal lymph nodes from which *M. bovis* was isolated, there were 12 (57.1%) which showed gross lesions.

Thoracic infection

Isolates of *M. bovis* were obtained from thoracic sites in 25 of 52 (48.1%) animals which had samples of their thoracic viscera cultured. Respiratory lymph node isolates were obtained from 22 of 46 (47.8%) pooled node cultures. However, this figure is biased because isolation of *M. bovis* was attempted only from visibly lesioned respiratory lymph nodes prior to July 1994. After this date all respiratory nodes were cultured and isolates were obtained from 14 of 38 (36.8%) deer sampled. Lesions were present in 17 of 22 (77.3%) respiratory node pools from which *M. bovis* was isolated.

Typical lung tubercles from five deer were submitted for culture (Figure 6-3). Four of the five furnished isolates of *M. bovis* (the fifth was a tiny 2 mm diameter lesion). Typical pulmonary lesions from seven other deer were not submitted for culture. Thirteen deer also had small grey homogeneous pulmonary nodules (often multiple), submitted for culture. From these, *M. bovis* was recovered in two cases.

Mycobacterium bovis was not recovered from nine of 11 (81.8%) of the pooled respiratory nodes submitted for culture from animals which had typical tuberculous lung lesions (tubercles or pleuritis). From seven of 22 (31.8%) cases from which *M.*

bovis was isolated from pooled bronchial nodes, there was an absence of gross tuberculous lung lesions.

The logistic regression analysis of the factors associated with the presence of the small grey pulmonary nodules resulted in the model presented in Table 6-IX. The only independent variable with a significant association was the presence of lungworm, and animals with these present were three times as likely to have the nodules present as those without lungworm.

Table 6-IX. Final logistic regression model examining the effect of lungworm presence on grey pulmonary nodules (n = 90)

Variable	Coefficient	SE	P	Odds ratio (95% CI)
Intercept	-1.31	0.31	<0.001	
Lungworm	1.10	0.49	0.024	3.0 (1.2 - 7.8)

Deviance = 103.1, $\chi^2_1 = 5.1$, $p = 0.02$

Many of the deer showed gross pulmonary parenchymatous lesions associated with the presence of lungworm. Gross lesions were characteristically found in the dorso-caudal lung lobes, and often appeared as thickened mottled parenchymatous areas up to several centimetres across, or as isolated red or brown discoloured lobules, which failed to collapse on opening the thoracic cavity. In some of these areas lungworm bodies, or exudate were found clogging the thickened bronchioles. Nine of these sites were examined histologically. They were characterised by bronchiolar smooth muscle hypertrophy, eosinophilic interstitial peribronchitis, and fibrosis of the interlobular septae. Bronchus-associated lymphoid follicles were hypertrophic, and occasional subpleural lymphoid hyperplastic areas were identified. Areas of atelectasis, emphysema, alveolar haemorrhage and haemosiderin deposition were also common. None of five subpleural lymphoid nodules examined histologically were found to contain lesions suspicious of tuberculosis or to contain AFB.

Logistic regression analysis of the factors affecting the presence of small fibrous pulmonary tags found none of the independent variables i.e. infection status, lungworm presence or sex, to be significantly related, in the 75 cases included in the analysis.

Of the 26 deer with *M. bovis* isolated from the thoracic contents, there were 11 which showed evidence of tuberculous pleuritis, either active or inactive. Five of

these cases were wild deer. In every case *M. bovis* was isolated from the respiratory lymph node pool, and in only one instance were gross lesions not present in at least one respiratory lymph node. In nine cases the pleuritis was associated with the presence of subpleural lung tubercles, but these were not always found in the same location as the pleuritis. Active cases were characterised by the presence of fleshy hemispherical translucent outgrowths up to 1 cm in diameter (“grapes”), which protruded from the lung surface. These were more common over the diaphragmatic lung lobes. In two cases where histological examination was performed the lesions were described as pleural villous hyperplasia, and were comprised of a mixture of lymphocytes, macrophages, neutrophils and plasma cells, with the occasional clump of denser cell aggregation in which some epithelioid cells were visible. No AFB were seen in either of the two pleural lesions examined. In three cases which were of apparently longer duration, the pleuritis had resolved and adhesions had formed between the visceral and parietal pleura in the mid to cranial thorax. Ten of the 11 deer with pleuritis were males, and although this relationship with sex was not statistically significant (Fisher’s exact 2-tailed test, $p = 0.178$, odds ratio $0.58 < 6.67 < 336.7$), it was suggestive that males were more likely to develop pleuritis.

Mesenteric lymph nodes

Mesenteric node lesions, usually singular, ranged in size from 1 to 10 cm in diameter, and were found along the length of the small intestine, from the abomasum to the ileocecal junction. Four mesenteric node lesions were found in one deer, which also had the largest tuberculous mesenteric nodes. In three cases from which *M. bovis* was isolated from mesenteric nodes there were no gross lesions, nor sites from which *M. bovis* was isolated elsewhere in the animal. In another three cases with gross mesenteric lesions there were no gross lesions elsewhere in the animal, but *M. bovis* was isolated from the oropharyngeal tonsil of one, oropharyngeal tonsil and medial retropharyngeal lymph node of another, and from the oropharyngeal tonsil and respiratory lymph nodes of the remaining deer. One suspicious 1 mm ileocaecal lymph node lesion was found in a deer with a large tuberculous medial retropharyngeal lymph node, but culture of this small lesion failed to isolate *M. bovis*.

Peripheral lymph nodes

Of the 98 deer with peripheral nodes examined there were eight with suspicious lesions which were submitted for culture. From these, isolates of *M. bovis* were recovered from four deer. Deer 2730 had gross lesions of all head nodes except one mandibular and one parotid node. The cervical lymphatic chain was grossly tuberculous, as were the caudal cervical nodes. The lungs showed miliary disease superimposed on an older extensive tuberculous pulmonary lesion. The abdominal viscera from this animal were unavailable for examination. Of the other deer with infected body nodes, one showed a mandibular lesion and also had an infected oropharyngeal tonsil, another had an infected popliteal lymph node and also had an infected oropharyngeal tonsil, and the remaining one showed an infected popliteal lymph node and also had a tuberculous respiratory lymph node.

Other sites

The liver from Deer 2730 contained a single 5 mm diameter tubercle, and was the only gross hepatic tuberculous lesion found in any deer. The hepatic parenchyma contained numerous microscopic granulomatous and pyogranulomatous foci. Occasional deer were found to have pinhead-size pale foci scattered over the surface of the liver, but histologically these were characterised by fibrosis and eosinophil accumulation, suggesting a parasitic origin. No gross kidney or splenic lesions were seen in any deer.

Gross versus bacteriological findings

Tuberculosis was provisionally diagnosed at necropsy by the identification of typical lesions. Sixteen of the 58 (27.6%) culture positive deer showed no typical gross tuberculous lesions. In these 16 deer, isolates of *M. bovis* were recovered from the oropharyngeal tonsil in 14 cases (which were associated with infection of the medial retropharyngeal lymph node in four instances, and an infected grey pulmonary nodule in one case), and the nasopharyngeal tonsil in two cases (with one of these animals also having an infected medial retropharyngeal lymph node).

Seventy two deer were provisionally diagnosed as tuberculosis-free at necropsy. From these animals *M. bovis* was isolated in nine cases (which comprise the majority of those mentioned above).

At necropsy, 45 deer were provisionally diagnosed as infected with *M. bovis*, but following culture, three cases were found to be culture-negative. Of the 3 without apparent infection, one had mineralised lesions in the respiratory lymph nodes and the lung, which histologically did not resemble tuberculosis, one other was a skin test reactor (farmed) which had a typical gross lesion in a mesenteric lymph node which was not submitted for culture, and the remaining animal had a purulent tonsillitis.

In another 45 cases there was a suspicion of infection with *M. bovis*, but of these only seven deer were found to be culture positive. From these seven, isolates came from the oropharyngeal tonsils of each animal, and the lung of one case. Four of these seven culture-positive animals were under suspicion because they were skin test and lymphocyte transformation assay positive (Chapter 5), one case had residual pleuritic tags and atypical lung lesions, another deer had two 3 mm grey pulmonary nodules which were without central caseation (but from which *M. bovis* was isolated), and the remaining animal had small fibrous tags on several lung lobe margins. Suspicious lesions in the remaining 38 deer from which isolates were not obtained were comprised of approximately equal numbers of small grey lung nodules, small fibrous tags on the lung lobe margins and tonsillar crypt lesions. Other lesions which occurred in one deer each were: a focal lesion in a bronchial lymph node; a small focal mesenteric lesion; a 3 cm diameter pulmonary adhesion; and a peripheral node with calcospherulosis.

Association between lesion sites

Culture-positive oropharyngeal tonsils occurred concurrently with infected medial retropharyngeal lymph nodes in 17 of 33 cases with infected medial retropharyngeal lymph nodes (where corresponding records were available). There was a positive and significant association between infection in the medial retropharyngeal lymph nodes and the oropharyngeal tonsil ($\chi^2_1 = 6.21$, $p = 0.013$), with those infected in the oropharyngeal tonsil 4.8 (1.2 - 21.3) times as likely to have concurrent infection in the medial retropharyngeal lymph nodes as those without.

In the four animals with nasopharyngeal tonsil infection, two were also infected in the oropharyngeal tonsil, but there was no significant association between infection at these two tonsillar sites (Fisher's exact 2-tailed test, $p = 1.0$). However, three of the deer infected in the nasopharyngeal tonsil also had infection of the medial

retropharyngeal lymph nodes. The association between infection at these sites was found not to be significant (Fisher's exact 2-tailed test, $p = 0.28$, odds ratio $0.37 < 5.17 < 279.8$), but although limited by low power, the odds ratio of 5.17 was suggestive of a relationship.

In nine of 23 deer with thoracic isolates of *M. bovis* there was concurrent infection present in the oropharyngeal tonsil or medial retropharyngeal lymph nodes. This association was highly significant ($\chi^2_1 = 13.7$, $p < 0.001$), with the odds of infection in the thoracic viscera being 12.4 (2.4 - 72.1) times as high in animals with oropharyngeal tonsil or medial retropharyngeal lymph node infection, as those without infection in these head sites.

Five of the 13 deer with gross mesenteric lesions also had lesions in the thoracic cavity from which *M. bovis* was isolated. However, contingency table analysis showed no significant relationship between the presence of mesenteric lesions and thoracic *M. bovis* isolates (Fisher's exact 2-tailed test, $p = 1.0$, odds ratio $0.2 < 1.1 < 5.1$). Seven of the 13 deer with mesenteric lesions also had isolates of *M. bovis* from either the medial retropharyngeal lymph node or the oropharyngeal tonsil. Although the association between these head sites and mesenteric lesions was not significant (Fisher's exact 2-tailed test, $p = 0.29$; odds ratio $0.50 < 2.38 < 10.87$), it was weakly suggestive of a relationship between orally acquired infection and lesions associated with the intestinal tract.

Excretion site sampling

Fifty three of the 58 infected deer had swab samples taken from the trachea, nasal cavity and the pharynx. *Mycobacterium bovis* was recovered from four pharyngeal swabs (7.5%), and in each of these cases the oropharyngeal tonsil was found to be infected. Deer 2730 was also excreting bacilli from the medial retropharyngeal lymph node via a suppurating sinus to the skin of the ventral neck (Figure 6-4). *Mycobacterium bovis* was also recovered from one nasal and one tracheal swab (1.9%) from Deer 2730. No other deer had isolates recovered from nasal or tracheal swabs. The faeces of 46 infected deer were submitted for culture, but only from the faeces of Deer 2730 was *M. bovis* recovered (prevalence = 2.2%). From none of 36 urine samples submitted for culture was *M. bovis* isolated, despite a sample from Deer 2730 being included.

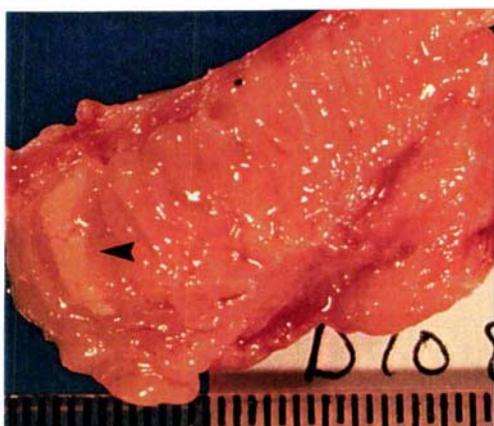


Figure 6-1. Longitudinal section of the oropharyngeal tonsil from a red deer. A small, elongated and soft caseous crypt lesion is present (arrowhead). Scale markers are in mm.



Figure 6-2. Sectioned oropharyngeal tonsil from a bovine showing multiple crypts containing firm caseous deposits similar to those of deer. These often shell out of the crypts following incision.

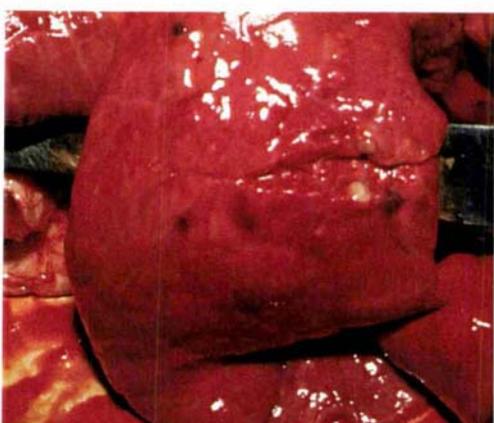


Figure 6-3. Typical small subpleural pulmonary tubercle on the intermediate lung lobe of a red deer. Small lesions without central caseation are difficult to distinguish grossly from lymphoid hyperplastic nodules.



Figure 6-4. Suppurating sinus on the ventral neck of Deer 2730. This was draining a large tuberculous medial retropharyngeal lymph node, and was associated with a terminal disease state.

Discussion

In no other country, has a prevalence of tuberculosis approaching 32% been reported in wild deer. The high level of infection seems to be associated with the presence of tuberculous possums in the same environment. *Mycobacterium bovis* was isolated from the oropharyngeal tonsil of 61% of the infected deer, and was the most frequently infected site of those examined. In nearly all cases, infection at this site was not associated with the presence of typical lesions. Typical tuberculous lesions were not found at all in 27.6% of the culture-positive deer, thus suggesting that the sensitivity of gross post-mortem/abattoir examination of infected animals will be limited.

It was not possible (for commercial and practical reasons) when recovering deer carcasses, to be able in many cases to collect all tissues on the sampling list. Thus in some instances only incomplete necropsies could be performed. The results must thus be interpreted in the context of the sampling limitations prevailing. Infection will not have been detected in these deer because of the incomplete examination of the carcass of many animals. Samples from pooled sites did not include all of the tissue from each site examined, so that if focal inapparent infection was present it may not have been included with the sample. Where pooled samples were made from paired sites, one of the pair was occasionally missing, destroyed by gun shot damage. There is also the risk that small lesions, containing very few bacilli may have failed to produce isolates. Notwithstanding these factors, it is believed that the prevalence figures provided are reasonably accurate, but moderately underestimate the true prevalence for the deer population and particularly for the various anatomical sites.

Prevalence of infection

The prevalence of tuberculosis (30 and 33%) in the two groups of wild deer was similar, despite the animals coming from populations a great distance apart. The prevalence was similar to that reported from earlier studies in the Central North Island endemic area. Between 1989 and 1993, 7 of 39 (18%) red deer were found to be infected by Nugent (1994). In December 1993, nine of 33 (27%) red deer taken from the Hauhungaroas were found to be tuberculous when examined more thoroughly (Nugent, 1994), but this figure was thought to underestimate the true prevalence, as the head and abdominal contents were not inspected in two thirds of

the deer. Nugent and Proffitt (1994) also reported infection in ten of 55 red and sika deer (18.2%) taken from the endemic area east of Turangi.

The prevalence of disease is much higher than that which has been reported in free-ranging deer overseas. In Britain, tuberculous wild and extensively managed deer have been found in the apparent absence of infected wildlife, but the prevalence of disease has been below 0.2% (Philip, 1989; Anon., 1990; Fletcher, 1990 and 1991). In areas where tuberculosis is endemic in badger (*Meles meles*) populations, British and Irish reports suggest that the prevalence of disease in deer may approach 5% (Dodd, 1984; MAFF reports, 1985 and 1986; Philip, 1989). Even though the estimates of disease prevalence from overseas may underestimate the true prevalence, they are unlikely to be wildly inaccurate. Schmitt, (1995) and B. Corso (pers. comm.) have also recently reported an outbreak in "managed" wild white-tailed deer (*Odocoileus virginianus*) in Michigan, in an area in which tuberculosis has been eradicated from cattle for many years. This suggests that the deer have been able to maintain the disease amongst themselves for a prolonged period in the absence of other hosts. However, in both Switzerland and Hawaii, where a low prevalence of tuberculosis in deer was known to occur whilst infected cattle herds were present, it is now believed that the wild deer are free of tuberculosis following elimination of the disease in livestock (Pastoret *et al.*, 1988; Nugent and Proffitt, 1994).

The point prevalence of tuberculosis in possums of the Hauhungaroa Ranges has been shown to vary, over a number of years, between 0.4 and 4.7%, (Nugent, 1994; Fraser *et al.* 1995; Pfeiffer *et al.*, 1995). The area surrounding Castlepoint is similar, in that tuberculous possums have been trapped there since the late 1970s, they are widespread and present throughout the year. In one intensively studied area possums have been found to have a tuberculosis point prevalence up to 20% and an annual prevalence of 10 to 20% (Pfeiffer, 1994; Jackson, 1995). The high prevalence of tuberculosis found in the wild deer of New Zealand may be due to sharing the environment with infected possums. This notion is given support by Nugent and Proffitt (1994) who found a high prevalence of disease in deer, associated with a focus of infection in possums on the forest-pasture margin, but with the tuberculosis prevalence in deer decreasing rapidly as the distance from the tuberculous possums increased.

In the central North Island endemic area, despite many wild deer being inspected at Game Packing Houses (GPH), no tuberculous deer have come from areas without infection being already present in possums (K. Paterson, pers. comm.). However, low prevalences of infection with tuberculosis in wild deer have been found in the absence of infection in possums. During a recent outbreak of tuberculosis in cattle, at Timahanga station, in the Rangitikei district (a non-endemic area), where a wildlife host was thought responsible, Nugent and Mackereth (1996) found only 1 of 128 wild deer examined was tuberculous. Tuberculosis was not found in any of 3,999 possums necropsied in the same area. In a similar incident, one of approximately 50 wild deer was found infected near another outbreak in cattle near Waikaka (Nugent 1994). No evidence of infection was found in thousands of possums necropsied in the surrounding area. In both these instances, as the large sample of possums examined failed to show evidence of tuberculosis infection, it was suggested that the wild deer may have been the source of infection for the cattle herds, but that in the absence of tuberculous possums the prevalence of infection in the deer was quite low.

Despite the absence of tuberculous possums or other wildlife hosts, it appears that tuberculosis may remain endemic, at a low prevalence, in some deer populations. The fact that the disease has apparently disappeared from deer in some countries, in the absence of other infected hosts suggests that wild deer alone are poor maintenance hosts of tuberculosis.

Transmission of infection

The low prevalence of infection in deer less than 13 months of age suggests that despite approximately 40% of their mothers being infected, few of the offspring acquire infection from their dam. Although neonatal deer are susceptible to infection with *M. bovis*, expression of the disease, which can after a time be quite severe (Robinson 1989; Anon, 1990), is limited in the early stage post-infection, by the immaturity and hyporesponsiveness of the cellular immune response (Watson and Gill, 1991; Griffin and Buchan, 1994), or possibly by the development of immune-tolerance (Veazey *et al.*, 1994). In farmed deer there are a number of reports suggesting that the prevalence of disease during outbreaks is lower in weaners than in older animals (Griffin 1988; Atkinson, 1993; Paterson, 1993; Whiting and Tessaro, 1994), suggesting that there may be some behavioural

characteristics operating which reduce the risk of infection in deer calves, despite having a close association with infected dams. It is possible that these younger deer may be too timid to approach possums, as has been observed with some older animals (Chapter 5). In addition the various routes of pseudo-vertical transmission are apparently not influential in deer.

The apparent high incidence of disease in yearling and mature deer suggests that most infection occurs in deer between one and four years of age. This supports the hypothesis that interspecific infection is acquired by older and bolder animals investigating sick tuberculous possums (Sauter and Morris, 1995a; Sauter and Morris 1995b).

At present deer population densities at both Castlepoint and in the Hauhungaroa Ranges, deer seldom form groups of more than a few animals, so the opportunity for intraspecific transmission is limited. The results suggest that transmission within these groups is not common, which is consistent with the low transmission rates usually observed in in-contact controls in experimental studies (Mackintosh and Griffin, 1994; Mackintosh *et al.*, 1995), and the low prevalence of tuberculosis in wild deer without contact with tuberculous possums (Nugent, 1994; Nugent and Mackereth, 1996).

Disease transmission by deer is likely to be associated with discharging sinuses from tuberculous deer in which the disease is under poor immunological control. Most severe outbreaks of disease in deer, have reported the presence of draining sinuses (Beatson *et al.* 1984; Robinson *et al.* 1989; Atkinson, 1993; Whiting and Tessaro, 1994; Mackintosh and Griffin, 1994). Coughing and associated generation of infectious aerosols is not likely to be the major route of transmission, as coughing is not a prominent sign in deer with pulmonary infection, as it is in cattle (Beatson and Hutton, 1981; Radostits *et al.*, 1994). Discharges from sinuses are likely to contain large numbers of bacilli which can potentially infect cohorts by direct contact and subsequent carriage of the bacilli onto the oral or nasal lymphoepithelial tissues. The victimisation of weak members of mobs of farmed deer, particularly by biting, will provide one effective transmission pathway. However, despite the occurrence of severe disease and suppurating sinuses in wild deer, the likelihood of transmission is reduced by the sickening deer parting company with others prior to death. Helicopter shooters have reported that animals in poor condition, affected by

severe tuberculosis are commonly located away from other deer (S. Gamble pers. comm.).

The recovery rate of *M. bovis* from the nasal, pharyngeal and tracheal swabs, urine and faeces was low, indicating that the majority of infected deer excrete few bacilli. The small number with severe disease excrete far higher numbers of bacilli. Similar results were reported by Corrin *et al.* (1987) where *M. bovis* was not isolated from 185 nasal swabs taken at regular intervals from 36 intratracheally inoculated deer. For swabs, the sensitivity of cultural techniques is limited by the small volume of the sample, and contamination by ingesta or normal flora of the site sampled. However, this is not seen as limiting the usefulness of the data, as in tuberculous humans with respiratory excretion, only a few individuals (with large numbers of demonstrable AFB in sputum) are believed to be responsible for the majority of transmission incidents (Rieder *et al.*, 1989). Thus by corollary, in deer if the swabs were positive, it should indicate that large numbers of bacilli were being excreted, and that the deer would likely be infectious to others. However, field evidence suggests that transmission amongst wild deer is at low incidence. Even in farmed deer many with severe respiratory disease, or individuals with suppurating sinuses need to be present before significant transmission will occur (Carter *et al.*, 1984; Beatson *et al.* 1984 Robinson *et al.* 1989; Atkinson, 1993; Whiting and Tessaro, 1994; Mackintosh and Griffin, 1994).

The recovery of *M. bovis* from three pharyngeal swabs taken from deer without severe disease, but with oropharyngeal tonsil infections, and the similar recovery from swabs of tonsillar fossae of the two deer reported in Chapter 5, strongly suggests that excretion from infected lymphoepithelial tissues is common. Ellsworth (1980) found that in pigs orally inoculated with *M. avium*, that the organism could be recovered later from faeces, swabs of the intestinal mucosa in the vicinity of intact Peyer's patches, and also from the tonsillar surface, thus suggesting that the lymphoepithelial sites infected by the inoculation, were excreting bacilli. Montgomery, (1987) also reported that tuberculosis was known to have spread in a number of deer herds where the only lesions found were in the retropharyngeal lymph nodes, thus suggesting that bacilli may have been shed from the oropharyngeal tonsils. As well as being capable of taking up mycobacteria, the lymphoepithelium also seems capable of shedding organisms following a latent

period after the initial infection. Nasal isolates of *M. bovis* have been recovered using sensitive cultural techniques, from a number of cattle, both naturally and experimentally infected, but without gross lesions in the lungs or upper respiratory tract (Neill *et al.*, 1988a; Neill *et al.*, 1988b; Neill *et al.*, 1989). Although the reports did not suggest a source of the organisms, it is quite possible that they may have been shed by the nasopharyngeal tonsils, which were not examined.

Age and sex effects on disease

The significant increase in the number of infected deer, showing no typical gross lesions, corresponding with an increase in age suggests that infected animals may be capable of resolving lesions over time. If the likelihood of infection were constant with age, and development of lesions a steady process, an increasing proportion of older animals would be expected to be found with gross lesions. Lesion resolution is a distinct possibility in this species where most animals show only evidence of single gross lesions at slaughter (Hathaway *et al.*, 1994), and in which lesions are not characterised by extensive fibrosis and mineralisation (Rhyan and Saari, 1995). Lesion regression/resolution has been observed in a number of other species (Gardner, 1922; Calmette, 1923; Rich 1951; Chapter 10), and there is no reason to believe that tissue repair processes in tuberculosis, where a protective immune response has been generated, will be different from other similar disease resolution processes. An alternative hypothesis, is that older animals are less likely to develop lesions when initially infected (such as those in Chapter 5). If this alternate hypothesis were correct, it would suggest that there are improvements in *M. bovis* protective cell mediated immune responses in deer over the age range of 1 to 10 years, which seems an unlikely phenomenon.

The suggestion from the data that male deer are more likely to show gross tuberculous lesions, including pleuritis, and develop severe disease has not previously been reported, but is in accord with observations in many other species in which males have been found to be less immunologically competent, particularly in cell-mediated immune responses associated with enhanced suppressor cell activity, decreased IL-2 production and lymphocyte mitogenesis, and generally resist a variety of parasitic, bacterial and viral infections less successfully than females (Castro, 1974; Ansar Ahmed *et al.*, 1985; Schalk and Forbes, 1997). However, there is no compelling evidence in the literature to suggest that males are

more susceptible to tuberculosis *per se*. Studies involving sex hormone administration and gonadectomy in laboratory animals have failed to produce consistent results with regard to demonstrating sex differences in pathogenesis or susceptibility (Lurie, 1955). It is more likely that, as most of the deer were killed during the period in which the males were in hard antler, that stress effects from intraspecific aggression, gathering and maintaining a group of hinds, and in some cases malnutrition may have played a role. Deer have been shown to be sensitive to the effects of stress and may respond by developing elevated circulating glucocorticoids, which are likely to increase the susceptibility of deer to a variety of diseases (Griffin *et al.*, 1988b; Griffin *et al.*, 1990), including tuberculosis (Thomson *et al.*, 1994; Thomson and Griffin, 1995).

Lymphoepithelial lesion distribution

Oropharyngeal tonsils were the most commonly infected site in the deer, but with crypt lesions being found in only half of the infected animals. The efferent drainage from the oropharyngeal tonsil is believed to pass directly to the medial retropharyngeal lymph nodes (Nickel *et al.*, 1981; Barrel and Simpson-Morgan, 1990), and accounts for the strong association between infection in the oropharyngeal tonsil and these nodes. This is the first report of the distribution of tuberculous lesions in any animal, which has identified the oropharyngeal tonsil as the most commonly involved site. In deer the medial retropharyngeal lymph nodes have been previously recognised as the most common site for lesions (Beatson *et al.*, 1984; Beatson, 1985; Wilcockson, 1986; Mackintosh and Griffin, 1994; Hathaway *et al.*, 1994). However in earlier work, tonsils have been cultured only in a few cases where they were examined (Brooks, 1984; Beatson *et al.*, 1984). Recently Mackintosh *et al.* (1995) have established an intratonsillar infection model which has been shown to closely mimic the natural disease. In these studies as few as 8 cfu have been instilled into tonsillar crypts, and produced disease in 50% of the inoculated animals. Taken together these data suggest that the most common portal of entry of *M. bovis* into deer may be via the oropharyngeal tonsil, with only small numbers of organisms needed to establish infection in the tonsillar crypts. The oropharyngeal tonsil, nasopharyngeal tonsil and Peyer's patches are lymphoepithelial tissues, in which the epithelium overlying the lymphoid follicles, contains specialised M-cells which are capable of endocytosing adherent mycobacteria, and presenting these bacilli to underlying dendritic cells and

macrophages (Fujimura, 1986; Momotani *et al.*, 1988). In this way mycobacteria and other antigens are presented for processing by the immunocytes underlying or within the epithelium.

From the tonsil, bacilli disseminate to other sites, principally the draining medial retropharyngeal lymph nodes. However, in contrast to other studies, the medial retropharyngeal lymph nodes showed a low prevalence of infection, and a high proportion showed no gross lesions, despite the presence of *M. bovis* in the nodes or in adjacent tonsillar tissues. This suggests that infection by possums may involve low numbers of organisms, that lesion development is less likely to occur, or that with a long duration of infection there is time for lesion regression and either destruction or induction of dormancy in bacilli.

Mackintosh and Griffin (1994) reported that gross tuberculous lesions of the tonsils were uncommon despite inoculation of *M. bovis* directly into the tonsillar crypts. The observations of this study also suggest that at least half of the deer with oropharyngeal tonsil infection show no gross lesions of the tonsil. However, the significant association between oropharyngeal tonsillar lesions and the infection status of the animals suggests that at least a proportion of the tonsillar lesions will be tuberculous in origin. However, the relationship between infection of the tonsil and lesion occurrence was found to be not significant, but could be explained by some lesions containing too few bacilli to be successfully isolated. This hypothesis is supported by finding a significant association between infection of medial retropharyngeal or oropharyngeal tonsil, and tonsillar lesions, with those infected in the lymph node or tonsil being twice as likely to show tonsillar crypt lesions as those without infection. Little can be said about the types of gross lesions which are caused by tuberculosis infection of the tonsil, except that they are common, occupy the crypts and take a variety of forms which are grossly indistinguishable from lesions caused by a variety of bacteria or reactions to foreign bodies (Payne and Derbyshire, 1963; Rohonczy *et al.*, 1996).

The observation that infection is often found in tonsils in which there are no visible lesions suggests that they, in common with other lymphoepithelial sites, such as the nasopharyngeal tonsil or Peyer's patch, usually remain free of grossly visible inflammatory responses when infected with mycobacteria, unless subjected to challenge from large numbers of bacilli (Griffith, 1907; Calmette 1923; White and

Minett 1941; Glover, 1941; Payne and Rankin, 1961). Furthermore this suggests that these sites are involved in immunological tolerance to antigens and/or have local suppressive responses which limit the potential for lesion development (Bienenstock, 1985), hinder clearance of the organism and allow development of a carrier state. Definitive histological and ultrastructural studies of the tuberculous tonsil are required to clearly define the range of pathology and movement of bacilli associated with *M. bovis* infection.

The nasopharyngeal tonsil was found to be infected in four cases, in three of which it was likely to have been the primary site of infection. This is the first time infection of this site with mycobacteria has been reported in any species. Although no significant relationship with medial retropharyngeal lymph node infection was found it is likely that one does exist, as the efferent drainage from the caudal nasopharyngeal area is believed to pass to the medial retropharyngeal lymph nodes (Nickel *et al.*, 1981; Barrel and Simpson-Morgan, 1990). The nasopharyngeal tonsil is situated in the roof of the nasopharynx, such that antigenic material trapped after inhalation into the nasal cavity, is likely to be swept by mucociliary action over the surface of the organ, prior to swallowing directly from the nasopharynx. The nasopharyngeal tonsil is likely to be the only mucosal site in the respiratory system of ruminants where particulate antigens can be taken up and presented to immunocytes (Chen *et al.*, 1989). The small number of isolates from this tonsillar tissue suggests that infection caused by aspiration of large airborne particles containing *M. bovis* which are likely to lodge in the nasal cavity, is uncommon in deer.

The significant association between head and thoracic infected sites suggests that after primary tonsillar infection that there is rapid haematogenous dissemination to the lungs. Calves fed contaminated milk have been shown to develop lesions in the lungs (Edwards, 1937; White and Minett, 1941), suggesting that early post-primary haematogenous dissemination from lymphoepithelial tissues to sites of predilection is common. Dissemination of mycobacteria from lymphoepithelial sites, via the bloodstream, may occur immediately following infection if the dose of bacilli overwhelms the local phagocytic defence mechanisms (Payne and Rankin, 1961), or may occur within weeks of the initial infection as bacillary numbers rise and some escape the lesion in migrating macrophages (Duchaine, 1938, cited by Francis,

1958; Fok *et al.*, 1976). The lungs have been found to contain a large population of intravascular macrophages capable of engulfing bacilli (Carrasco *et al.*, 1996), and are also known to sequester large numbers of circulating leucocytes (Hay and Cahill, 1982) and to retain damaged (infected) cells (Thakur *et al.*, 1977), both mechanisms which can lead to the development of pulmonary disease.

Pulmonary lesions

The presence of grey subpleural pulmonary nodules was significantly related to the presence of lungworm only, but their occurrence presents a diagnostic dilemma as these can be caused by both lungworm and *M. bovis* infection. Isolates of *M. bovis* were recovered from 2 of 13 of these lesions submitted for culture, but of five examined histologically, all resembled typical lymphoid hyperplastic nodules. Development of lymphoid hyperplastic nodules has been described following pulmonary infection in guinea pigs with tuberculosis (Gardner, 1922), but not in other species. Similar subpleural lesions, composed of hyperplastic lymphoid tissue have been reported in cattle, where they are associated with previous exposure and acquired immunity to lungworm. The nodules are believed to be the end stage of a localised acute inflammatory response to parasitic antigens (Breeze, 1985). In a study of thoracic pathology in Scottish red deer, lesions associated with *Dictyocaulus* spp. infection were found commonly in both wild and farmed deer (Munro and Hunter, 1985). Lymphoid tissue had formed in peribronchiolar, interlobular and subpleural sites, which was in accord with the findings of this study, and other experimental infections with *Dictyocaulus viviparus* in deer (Corrigal *et al.*, 1982; Munro, 1988). No animals in this study were found to have large numbers of parasites or associated lesions which would have significantly compromised the health of the individual, but the parasitic lesions were common and required close examination to rule out the possibility of them being tuberculous granulomas.

In retrospect, it is believed that careful macroscopic examination should be able to differentiate small grey homogeneous nodules into those with a granulomatous appearance and those with a lymphoid follicular appearance. If the lesions are mature lymphoid nodules, the follicles they contain will give the cut surface a granular appearance, which is not apparent in the granulomatous tuberculous nodule. When the centre of a nodule is necrotic a tuberculous granuloma should

always be suspected, but they can be quite small and nestled in amongst the parenchyma, and thus difficult to find. These small tuberculous pulmonary lesions are not routinely looked for at DSP nor GPH inspections, and their undetected presence will lower the sensitivity of inspection procedures as there will not always be corresponding gross lesions in the respiratory lymph nodes. Further research is necessary to clarify the relationship between gross lesion appearance and tuberculosis infection status.

Small fibrous tags on the margin of the diaphragmatic lobes, especially the ventral area, are common in ruminants, and are believed to be due to mechanical irritation caused by constant movement and weight of abdominal viscera nearby (G. Wobeser, pers. comm.). It was initially suspected that some of these may have been associated with pulmonary tuberculosis, however this was found not to be the case. However, small fibrous tags on the large broad surfaces of the lung lobes are likely to be residual foci from larger pleuritic lesions, possibly of tuberculous origin as they were also found overlying lymphoid nodules and small tubercles, presumably the result of localised inflammatory responses to lungworm or *M. bovis* infection.

Tuberculous pleuritis is believed to be associated with small foci of sub-pleural pulmonary infection, and escape of bacilli to the pleural cavity (Talavera *et al.*, 1994; Barnes *et al.*, 1994). This seemed to be true in the current series of animals where in nine of the 11 cases small sub-pleural tubercles were found. In humans pleuritic lesions are believed to develop as a local manifestation of protective delayed-type hypersensitivity responses (Talavera *et al.*, 1994; Barnes *et al.*, 1994), and this may account for the low numbers of bacilli found in such lesions, and the non-progressive nature of associated pulmonary lesions. Adhesions, the end result of tuberculous pleuritis, were uncommon and restricted to the cranial and intermediate lung lobes where there is less movement to disrupt their formation. Although adhesions resulting from caudal thoracic trauma and fractured ribs have been found in up to 2.2% of slaughtered farmed deer (Wilcockson, 1986; Hathaway and Selwyn, 1991; Selwyn and Hathaway, 1992), none of this nature were found in the deer examined.

Other sites

In each case where there was involvement of a peripheral body node, infection was found at another site. The oropharyngeal tonsil was also involved in two cases, the

respiratory lymph nodes in another case, and generalised disease in another. This suggests that early post-primary bacillaemia, as well as allowing spread to the lungs, may also allow lesions to develop, in what appear to be peripheral predilection sites, such as the popliteal and caudal cervical lymph nodes (Hathaway *et al.*, 1994). Brooks (1984) reported the occurrence of single lesions in caudal cervical and popliteal nodes of 3 of 15 in-contact controls in an experimental intratracheal inoculation trial. This lesion distribution was suggestive of early haematogenous dissemination subsequent to infection at an unidentified primary site, possibly tonsillar. Although skin wounding/contamination, with subsequent infection of the draining lymph node has been hypothesised as a cause of peripheral nodal lesions (Mackintosh and Griffin, 1994), there is no field evidence available to support such a notion.

Although a significant association between head site infection and bowel-associated lesions was not established in our data, a significant association was found by Hathaway *et al.* (1994) when examining the slaughter records of 668 lesioned tuberculous deer. This suggests that head-associated infection and bowel infection occur at approximately the same time, as within weeks of infection an immune response is generated (Griffin *et al.*, 1993a), which if protective, will limit the likelihood of subsequent lesion development despite the probability that some bacilli will, at least intermittently, circulate in the bloodstream. From the present data, there was no indication that a significant association between infection in thoracic sites and the bowel-associated sites existed, which suggests that lung and bowel infection do not occur simultaneously, and that pulmonary lesions do not commonly result in sufficient excretion of bacilli to result in bowel-associated lesions.

The medial retropharyngeal node is the most common site of tuberculous lesions, outside of the thorax, in cattle (Francis, 1958; Crews, 1991; Neill, 1994). This suggests that the acquisition of infection by the tonsil may be one of the more important routes of natural infection in both cattle and deer. The inapparent infection of tonsils may also be a significant contributor to the occurrence of non-lesioned cattle and deer reactors (to intradermal or blood tests for tuberculosis) examined at slaughterhouses. This is a particularly common phenomenon in deer where 79.9% of reactors to intradermal testing fail to show lesions at abattoir

inspection (Hathaway *et al.*, 1994). This high percentage of 'no gross lesion' reactors has typically been attributed to high levels of non-specific reactivity (as was probably the case in some of the farmed deer examined in this study). However, the results of this research suggest that the proportion of truly infected deer which show no gross lesions can be considerable.

Conclusions

This study has shown the importance of lymphoepithelial tissues as primary sites for the establishment of tuberculosis infection, and for the subsequent excretion of organisms. These sites, such as the oropharyngeal tonsil and nasopharyngeal tonsil usually remain free of gross lesions, or if they do develop lesions they are impossible to classify as tuberculous from gross appearance. The overall impression of *M. bovis* infection of wild red deer, is one where the disease process is kept under control by appropriate cell-mediated immune responses, and with little bacillary excretion from most animals. The findings of this study are in agreement with those of Griffin and Buchan (1994) who concluded that deer are not innately highly susceptible to *M. bovis* infection as has been previously suggested by McDiarmid (1960). However, as with farmed deer, stress may precipitate serious disease, particularly in males during sexually active periods.

The high prevalence of tuberculosis in free-ranging deer associated with the presence of infection in possums suggests that substantial reductions in the prevalence of disease in deer will occur if eradication of the disease from possums is achieved. However, despite the apparent low transmissibility of disease amongst wild deer, other reports suggest that deer may maintain a low prevalence of infection, in the absence of other infected hosts which can transmit infection to them. However, this reservoir host status may be a density dependent phenomenon. This implies that eradication of tuberculosis from wildlife populations in New Zealand is unlikely to be achieved without the incorporation of deer tuberculosis control measures, as deer have the potential to reintroduce infection into possum populations free of the disease.

Acknowledgments

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The PhD candidate was responsible for the design and conduct of this research. All necropsies and sample collection were either carried out by the candidate or under his direct supervision. Data analysis and preparation of the manuscript were also carried out by the candidate.

CHAPTER 7

***MYCOBACTERIUM BOVIS* INFECTION IN NEW ZEALAND HEDGEHOGS (*ERINACEUS* *EUROPAEUS*)¹**

¹ Published as I. W. Lugton, A. C. Johnstone and R. S. Morris. (1995). New Zealand Veterinary Journal, 43(7), 342-345. This chapter contains additional information on 2 later cases not presented in the published article.

Abstract

One hundred and fifty five wild hedgehogs from the Wairarapa, a bovine tuberculosis endemic area of New Zealand, were examined for lesions suggestive of tuberculosis. Forty one animals with suspicious lesions had samples submitted for culture. *Mycobacterium bovis* was recovered from five animals with visible pulmonary lesions. A presumptive diagnosis of tuberculosis was made in one other animal which had acid fast organisms in subcutaneous lesions. The gross and histopathological appearance of the lesions are described and the significance discussed in the light of previously reported experimental and captive animal infections. It is likely that infection arose from the scavenging behaviour of hedgehogs. The moderate prevalence (3.9%) of tuberculosis in these animals combined with their small home ranges may allow them to be used successfully in wildlife surveys to pinpoint the locality in which tuberculous possums have died.

Introduction

The hedgehog, although a common mammalian inhabitant of much of the New Zealand countryside and its urban areas, has not previously been confirmed in New Zealand as a host for bovine tuberculosis. This is despite the fact that several wild hedgehogs have been found naturally infected with mycobacteria (Stockdale, 1975; Matthews *et al.*, 1981; Schliesser, 1985; Brockie, 1990). As hedgehogs are not only scavengers but are also eaten by some predatory species such as badgers, ferrets and pigs (Bradbury, 1974; King, 1990), all of which are, or may be, involved in the epidemiology of tuberculosis in one or more countries, an insight into the disease status, and associated pathology, of the species is of value. This paper details the findings of such a study.

Materials and Methods

Wild hedgehogs were initially collected from the Castlepoint area in the Wairarapa district of the North Island. This was part of a cross-sectional study of wild mammals in 1994 which aimed to determine the prevalence and strains of *Mycobacterium bovis* infecting wild mammals other than possums in the area. The investigation was designed to complement the results of an ongoing longitudinal study of tuberculosis in possums and domestic stock in the vicinity (Pfeiffer, 1994). Further hedgehogs were later obtained from the southern end of Lake Wairarapa. The period of collection extended from early 1994 to mid 1995. All but two of the animals (from outside the Wairarapa) were collected from areas known to be endemic for bovine tuberculosis.

Animals were predominantly captured in padded leg-hold traps baited with meat, which had been primarily set to catch predatory mammals such as ferrets and cats. Some were also caught in cage or box traps baited with meat or broken hens' eggs. Other hedgehogs were gathered opportunistically as fresh road casualties or supplied frozen by local farmers where they had been caught as non-target species during possum trapping operations. Live hedgehogs were humanely killed by the injection of intraperitoneal sodium pentobarbitone, identified and later weighed.

The necropsy procedure involved securing the carcass by clamping anchored tissue forceps onto the nose, and then removing the spineless skin of the ventral body

whilst noting the appearance of the numerous subcutaneous lymph nodes. Mandibular and popliteal nodes were examined before disarticulating the clavicle, cutting through the axillary regions and splaying the forelegs. The ventral surface of the thorax and attached strip of abdominal musculature was then removed. Retropharyngeal lymph nodes were examined, and the lungs inspected and palpated. Abdominal contents were checked, paying particular attention to the colonic and jejunal lymph nodes. Samples of lesions for mycobacteriology were collected at this stage if suspicious lesions were present. The stomach was opened last and the contents removed to aid in the estimation of volume and characterisation of the contents. Animals in which tuberculosis was suspected were usually then preserved whole in buffered formol saline for later histopathological examination. Standard measures were taken during the examinations to avoid cross-contamination of mycobacteria between the animals necropsied.

Following the necropsy, maturity, sex, physiological status, amount of fat reserves, estimated stomach volume and contents, gross lesions and samples retained were recorded. Tissues removed for bacteriology were stored in sterile plastic containers at -84°C and later submitted to the AgResearch tuberculosis laboratory, Wallaceville, for culture, using the techniques described by (Buddle *et al.*, 1994). Fixed tissues from culture positive animals were later submitted to the Ministry of Agriculture and Fisheries, Batchelar Animal Health Laboratory, Palmerston North, or to Dr. Gary Wobeser, wildlife pathologist from the University of Saskatchewan, Canada, while on sabbatical leave in the Department of Veterinary Pathology and Public Health, Massey University, Palmerston North. Paraffin-blocked tissue prepared from this material by routine histological processing was sectioned and stained using the haematoxylin/eosin and Ziehl-Neelsen methods. Selected sections were also stained by Gram's method.

Results

One hundred and fifty seven hedgehogs were necropsied, comprising 93 females (including 17 juveniles) and 64 males (including 10 juveniles). One hundred and eleven of the hedgehogs had some food in their stomachs, with details recorded in 97 cases. There were seven (7.2 %) occurrences of mammalian tissue (meat, fat or

fur), four of these being bait material. Three other stomachs (3.1 %) contained maggots.

There were 41 animals with suspicious lesions, from which tissues were submitted for mycobacterial culture. These tissues consisted of 13 nodular lung lesions, one hepatised pulmonary lesion, one lung cicatrix, 21 enlarged mesenteric lymph nodes, three focal liver lesions, two skin lesions, one focal splenic lesion and one sample of necrotic peritoneal adipose tissue. Three enlarged mesenteric lymph nodes also had sub-samples submitted for routine aerobic culture. One normal mesenteric lymph node was also submitted from a non-lesioned hedgehog. *Mycobacterium bovis* was isolated from five lesions from cases 2 to 6, and in which acid fast bacilli (AFB) were also demonstrated. A tissue sample from case 1 contained AFB, but although submitted for mycobacteriology failed to grow *M. bovis*. The apparent prevalence of *M. bovis* infection was 3.9%. *Mycobacterium avium* was isolated from an enlarged jejunal lymph node from one hedgehog (0.6 % prevalence). The individual cases of tuberculosis are described below.

Case 1, a mature female with poor fat reserves was caught on the roadside near Castlepoint on 1 April 1994. This road ran through a pine plantation, from beside which many tuberculosis reactor cattle have come. The lesions found at necropsy consisted of two 1 cm diameter, irregular creamy areas of caseation and inflammation in both the left and right flank. *Mycobacterium bovis* was not isolated from the lesions, but AFB were present in histological sections.

The second case was a mature male with good fat reserves caught on 19 May 1994 in the Castlepoint area. This animal was found in the immediate vicinity of a site from which a tuberculous possum had recently been captured. About 12 grey foci with irregular borders which did not protrude above the pleural surface were scattered in the pulmonary parenchyma. The lesions were 2-6 mm in diameter, firm and homogeneous on incision. Bronchial lymph nodes were enlarged.

The third hedgehog (case 3) found to have tuberculosis was a well-conditioned mature female. She was captured in mid-August 1994, on a property adjacent to the Castlepoint longitudinal study site. She was frozen prior to necropsy. The only lesion found was a single 5 mm nodule in the substance of the left diaphragmatic lung lobe which was initially detectable only by palpation. Incision revealed a firm,

even textured mass which showed a red discolouration, probably induced by the freezing.

Another tuberculous mature male (case 4), also in good condition, was trapped on the study site on 12 October 1994. The site of capture coincided with an area where several decomposing tuberculous possum carcasses had been found 8-11 months earlier. The lesions again were confined to the lungs, with six grey-coloured spherical nodules, up to 5 mm diameter, scattered over and bulging from the pleural surface. On incision these were of similar texture and general appearance to the lesions in case 2.

The next confirmed *M. bovis* infection was found in a mature male (case 5) captured near Lake Wairarapa on 15 January 1995. This emaciated hedgehog was affected with generalised tuberculosis. The lungs had a similar appearance to case 2, but with more lung parenchyma replaced by consolidated tissue (Figure 7-1). The left retropharyngeal lymph node was enlarged to twice normal size and was largely composed of yellow caseous material with some mineralisation. The medulla and pelvis of the right kidney contained an abscess which had caused thinning and atrophy of the cortex resulting in an irregular, mottled appearance of the surface. The left kidney was distended by urine retention resulting from a partially blocked urethra caused by the enlarged and abscessed right lobe of the prostate gland. The affected prostate gland had extensive yellow caseous foci scattered across the cranial aspect (Figure 7-2). The right mandibular lymph node was enlarged and the liver surface showed a small number of pin point sized grey foci.

The last confirmed case (6) of *M. bovis* was also from the Lake Wairarapa area close to the capture site of case 5. This was a female in poor condition which had recently finished lactating. Necropsy revealed a carcass with multiple lesions, which included a right retropharyngeal lymph node 10 times larger than normal, but without caseation (Figure 7-3). The colonic and jejunal lymph nodes were normal in colour, but twice their expected size. The lungs contained five grey spherical foci up to 6 mm in diameter, and several other areas of cicatrization on the surface (Figure 7-4). The caudal mediastinum also contained several pleural grapes up to 1 mm diameter, and the cranial mediastinal lymph nodes were enlarged to three times their normal size.

Case 7, a mature female in average condition, was trapped at the southern end of Lake Wairarapa in early December 1994. On necropsy this animal was noted to have jejunal and colonic lymph nodes three times normal size, but still retaining the usual grey-green coloration. *Mycobacterium avium* was later cultured from samples of these lymph nodes. A length of jejunum and associated lymphatic and pancreatic tissue was kept for histopathology, but no lesions suggestive of tuberculosis were visible histologically.

The histological appearance of the tuberculous pulmonary lesions was similar in all hedgehogs, characterised as focal but locally infiltrative, non-encapsulated and non-caseating granulomatous inflammation. The normal parenchyma was destroyed by the dense accumulation of macrophages and leucocytes. Macrophages were the dominant cell type with multinucleated giant macrophages (Langhans' type) being numerous throughout the reaction. Interspersed between the macrophages in smaller numbers were lymphocytes, plasma cells and occasional polymorphonuclear leucocytes.

Many of the smaller microscopic lesions had a close association with peribronchiolar tissue, while bronchiectasis was present in some of the larger lesions. Necrosis, mineralisation and fibrosis of tissue in the lung lesions was minimal or absent, except in case 6, where advanced fibrosis was a feature of the granulomas. Acid fast bacilli were present in moderate numbers in the lung lesions of case 3, were less frequent in case 4 and were infrequent in cases 2 and 5.

The bilateral subcutaneous lesions seen in case 1 were composed of severe narcotising pyogranulomatous panniculitis and myositis, with numerous AFB within the lesions.

In addition to the pulmonary lesions in case 5, tuberculous lesions were present in the left retropharyngeal lymph node, kidney and prostate gland. The affected lymph node was extensively damaged by coalescent areas of caseous and calcifying granulomatous inflammation with peripheral fibrosis. The cellular component of the reaction was otherwise similar to that described in the lungs.

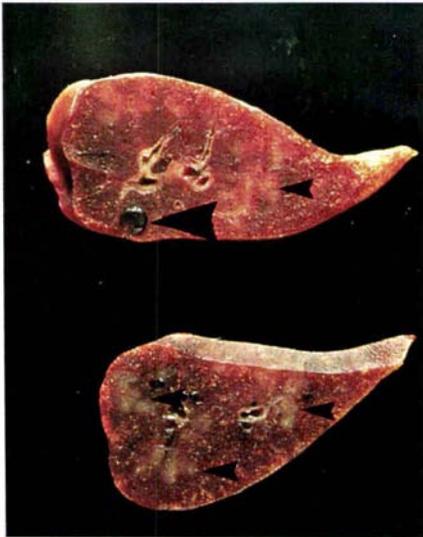


Figure 7-1. Lung of case 5 with confluent areas of consolidation (small arrows) caused by *M. bovis* infection. Pulmonary blood vessels are dilated (large arrow) due to thrombosis.



Figure 7-2. Tuberculosis in the prostate gland of case 5. There is diffuse fibrosis of the right lobe (large arrow) with multiple caseous foci (small arrows) throughout. The left lobe of the prostate gland (P) and the seminal vesicle (SV) are normal.

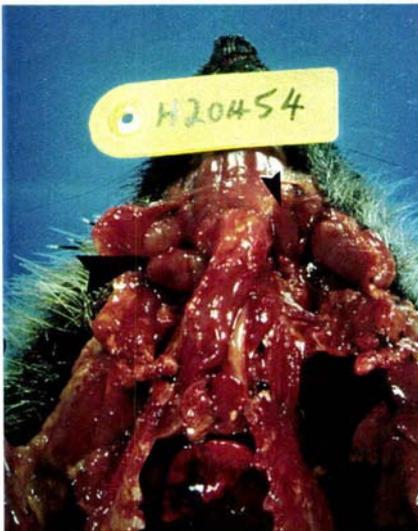


Figure 7-3. Ventral neck region of case 6 with grossly enlarged right retropharyngeal lymph node (large arrow) and the normal left counterpart (small arrow).

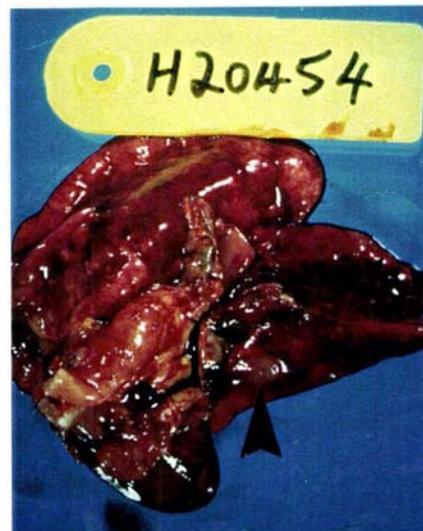


Figure 7-4. Lung of case 6 with a large granuloma protruding from the lung surface (large arrow) and areas of pleural cicatrization (small arrows).

The normal architecture of the prostate was destroyed by multiple caseating tuberculoid granulomas, similar to those in the lymph node but with the additional change of extensive fibrous reaction of the gland interstitium. Both kidneys had non-caseating pyogranulomatous pyelitis, with many neutrophils present in the reaction of the left kidney. In the right kidney, the granulomatous papillary lesion extended to involve the corticomedullary tissue. The tubules of the overlying cortex were atrophic with marked interstitial fibrosis.

Case 6 had many more histologic lesions than those visible grossly. There was severe granulomatous lymphadenitis with extensive fibrosis and many large giant cells in the left mandibular, right retropharyngeal, right prescapular, left axillary, bronchial, mediastinal, jejunal and colonic lymph nodes. There was no necrosis visible in any of the sites. The liver contained numerous randomly distributed aggregations of epithelioid cells. The thymus and one of the salivary glands also contained granulomas. The pleural grapes were composed of a fibrous matrix infiltrated with numerous small granulomas containing central epithelioid cells, occasional giant cells and a marginal ring of lymphocytes and plasma cells.

Acid fast bacilli were present in moderate to high numbers in each of the non-pulmonary lesions in all animals, except case 6 where there were only a few seen in the left mandibular lymph node. No AFB were seen in any of the grossly normal tissues of the hedgehogs.

Non-tuberculous lesions identified histologically in the tuberculous hedgehogs included; bronchial lymph node hypertrophy (case 2), *Crenosoma erinacei* infestation of bronchi (case 2 and 6), granulomatous submandibular lymphadenitis related to vegetable foreign material (case 4) and multiple focal non-tuberculous granulomas associated with unspiciated liver flukes (case 5).

The routine aerobic culture of three enlarged jejunal lymph nodes produced growths of *Salmonella enteritidis* (phage type 9a), one of these nodes coming from a moribund animal which had succumbed to the infection. Granulomatous lesions, consistent with the presence of salmonellae were also found in four other jejunal nodes. The lungs of seven animals were dotted with pin-point grey foci which were barely palpable. These tiny granulomas containing macrophages, lymphoid cells and eosinophils, were associated with the presence of the common lungworm, *Crenosoma erinacei*.

Discussion

This paper describes what are believed to be the first confirmed isolations of mycobacteria from wild hedgehogs in New Zealand. There have, however, been other unconfirmed cases of tuberculosis in hedgehogs in this country which have arisen in the course of research or wildlife surveys for tuberculosis. In a post-mortem leptospirosis survey of 155 hedgehogs from the southern part of the North Island in 1957, Brockie (1958) found one animal with pulmonary lesions containing AFB. This may in fact be the earliest record of wildlife tuberculosis in New Zealand, eclipsing the infected feral pigs found in 1964 (Ekdahl *et al.*, 1970). If so, it would suggest that tuberculosis was probably already present in possums in the lower North Island at that date. Beatson found presumptive tuberculosis in a West Coast hedgehog in the early 1970s (Stockdale, 1975), although it is uncertain as to whether the presence of *M. bovis* was confirmed due to loss of records. G. Atkinson (pers comm.) similarly found a Wairarapa hedgehog with AFB in pulmonary lesions in 1979.

In Britain, *M. bovis* was recovered from a hedgehog found in Regent's Park, London, in 1932 (Hamerton, 1933). This animal died from the disease after 5 weeks confinement in the Regent's Park Zoo. Lung lesions were described by Hamerton as being of the "grey hepatisation" type and contained large numbers of AFB. It was believed that the hedgehog may have become infected through drinking infected cow's milk. Hamerton (1936) also reported the death of another tuberculous hedgehog after nearly a year's captivity in the zoo. *Mycobacterium bovis* infection was not confirmed but lesions were found in the lungs and intestinal tract. Schliesser (1985) also remarked that occasional cases of bovine tuberculosis were found in hedgehogs in Germany prior to the eradication of this disease.

Mycobacterium avium type 2 has also been recovered from the mesenteric lymph nodes of a wild hedgehog from the Berkshire Downs (Matthews *et al.*, 1981). This was one of five non-lesioned animals cultured as part of a research survey. The isolation of *M. avium* from an enlarged jejunal lymph node in the current study may indicate that this common environmental bacterium may be capable of causing lesions in this species.

Griffith (1907) subjected sixteen hedgehogs to experimental *M. bovis* infections during the Royal Commission into tuberculosis. Six animals infected parenterally with doses of up to 1 mg of culture, showed a predisposition to caseous lesion development in the local lymph nodes and small non-caseating granulomas forming in the liver, spleen, kidney and lungs. Ten other animals were given doses of up to 10 mg of culture orally. Of these four developed no gross or microscopic lesions. The six remaining showed caseous lesions in the retropharyngeal or cervical lymph nodes (n = 4), or small grey lesions in the lungs (n = 3), one animal developed haemorrhagic intestinal lesions and another had small omental lesions with calcification. Although AFB were seen in some of the mesenteric lymph nodes these were not associated with gross lesions. Many of the hedgehogs died a month or two after inoculation, but it was not made clear whether the deaths were the result of tuberculosis or poor husbandry.

There is a striking similarity of gross lesions in nodal and pulmonary sites between Griffith's experimental cases (Griffith, 1907) and the present series of tuberculous hedgehogs. The development of caseating granulomas (Koch-type reactions) at the former site, in conjunction with the more florid infiltrative and non-caseating pulmonary granulomas is an unusual feature of the disease in this species. Mammals which exhibit lesions of the caseating type usually have a well developed cell-mediated immune response (Thorns *et al.*, 1982). This class of reaction is characteristic of the disease in primates, ungulates, guinea pigs and rabbits and was representative of the nodal and prostatic lesions of the generalised case in this series. In mammalian species in which cell-mediated responses are difficult to demonstrate or slow to develop, the lesions of tuberculosis are usually progressive and destructive, non-caseating and infiltrative. The lung lesions of each of the hedgehogs in the present series, although perhaps less invasive and more slowly progressive, were of the latter type. The degree of fibrosis present in the nodal and prostatic lesions of Case 5 suggested that these changes were considerably older than those in the lung of the same animal. No ready explanation can be offered for the striking morphological differences of the pulmonary and non-pulmonary lesions. Thorns *et al.* (1982), suggested that the essential character of tissue change in response to *M. bovis* is reliant on the presence and/or intensity of the cell mediated-immune response. The observed dichotomy of lesion types may thus be due to differences in cellular immunity acting at the time each lesion developed, or

more likely through differences in the nature of the immune response in various sites (Barnes *et al.*, 1989).

Thorns *et al.* (1982) also experimentally infected five hedgehogs by intraperitoneal injection with a dose of 500-5000 *M. bovis* organisms. When these animals were killed at 40-60 days no gross lesions were visible, although there were microscopic granulomas in the kidneys containing AFB, necrotic cells, polymorphonuclear leucocytes and epithelioid cells. Acid fast bacilli were also seen in the lungs and spleen with no associated lesions, and mycobacteria were additionally cultured from the liver. Interestingly, the hedgehogs in this study failed to show any specific measurable immunologic responses to the presence of the mycobacteria. It was proposed that the low body temperature, between 33 and 38 °C, may have limited the pathogenicity of the bacilli, but this seems unlikely, given the severity of the disease in brushtail possums (*Trichosurus vulpecula*) which have an average body temperature of 36.2 °C. It appears that the hedgehog is a naturally moderately resistant species which can however mount an effective cell-mediated immune response to *M. bovis*. Case 6, with the widespread fibrotic granulomas containing few AFB and pulmonary scarring, is probably a good example of an individual, massively challenged, but mounting an effective response, so that lesions ceased to progress or resolved.

From these reports and more recently encountered cases it appears that the usual manifestation of tuberculosis from oral infection in the hedgehog is one of grey coloured pulmonary granulomas, which are slow growing and contain low to moderate numbers of AFB. Retropharyngeal and cervical lymph node involvement can be expected in some animals, with nodes showing enlargement, caseation and possible mineralisation. Mesenteric lymph node lesions are not a common feature, but small granulomas may develop in the kidney, liver and spleen. Enteric, severe renal and prostatic involvement are possible and may provide a route of excretion in occasional cases. However it seems most unlikely that hedgehogs would represent a significant source of infection.

As hedgehogs will eat almost any animal substance, including fly maggots (Brockie, 1959), it seems quite likely that in endemic areas they will be exposed to *M. bovis* infection from the investigation of decomposing carcasses, particularly those of possums. An unknown percentage will become infected and subsequently

diseased. The 3.9 % prevalence found in this study is probably an underestimate of the true prevalence, as it is likely that some hedgehogs will harbour *M. bovis* without evidence of gross lesions.

Infection is most likely to be by the oral route as hedgehogs are largely solitary animals except during brief mating encounters and while rearing young. Most lung lesions identified so far only appear likely to shed small numbers of AFB into the respiratory tract. As with many other species, the lung, due to its own physiologic peculiarity (especially the high oxygen tension) (Meylan *et al.*, 1992), large population of phagocytic cells and the passage of the entire circulating blood volume through its capillary network, is a site of predilection for lesion development. The bacilli probably enter the circulation following uptake and replication in macrophages, after initially being phagocytosed from the mucosal surface by lymphoepithelial cells of the tonsils and Peyer's patches (Wolf and Bye, 1984). Excretion of organisms via the urine, subsequent to kidney infection, is a possibility. Hedgehogs are known to dribble urine trails as they wander about the pasture (Brockie, 1990), but the probability of transmission either to other hedgehogs or to grazing herbivores by this route appears low. As the likely feeding ranges of hedgehogs, particularly the females, in New Zealand are small (e.g. between 1.9 and 3.6 ha) (Reeve, 1994), the discovery of a tuberculous hedgehog is probably indicative that a tuberculous animal of another species, such as the possum, has died in the vicinity at some time in the recent past. The moderate prevalence of tuberculosis, limited home range, and ease of capture of these animals in wildlife tuberculosis surveys may make them useful as an additional indicator species for endemic tuberculosis.

Hedgehogs are themselves prey for other species. Pigs and ferrets are known to prey on these animals, and their carcasses will also be scavenged by a variety of mammals and birds. In a recent study in Central Otago (Smith *et al.*, 1995), hedgehog remains were found in six of 140 (4.3%) ferret stomachs containing food. An earlier Manawatu investigation found an even higher occurrence of hedgehog tissues in ferrets (Roser and Lavers, 1976). Ingestion of hedgehogs by ferrets is clearly commonplace, and may well provide another avenue by which wild ferrets acquire infection. An unsuccessful predatory attack by a ferret, which are known to

commonly shed *M. bovis* in the saliva (Lugton *et al.*, 1995), on the hedgehog with the bilateral flank lesions, may explain this occurrence.

On the available evidence, it would seem that hedgehogs are yet another spillover host for tuberculosis in areas of New Zealand where the weight of infection is high, and that infection occurs (as in other scavenger species) by ingestion of infected carcasses. It appears most unlikely that infection would be self-maintaining in hedgehog populations in the absence of infectious food sources, in contrast to the situation in possums, which have been shown to be maintenance hosts.

Acknowledgments

The authors are grateful to the Animal Health Board for funding this project, and also the farmers of the Wairarapa who allowed us to trap on their land or who provided animal samples. The histopathological examination of some animals by Gary Wobeser was also much appreciated.

The principal author was directly involved in trapping many of the animals, and conducted almost all necropsies on the hedgehogs. Literature review, manuscript preparation etc. was also carried out by the PhD candidate. The majority of histological examinations were carried out by A. C. Johnstone.

CHAPTER 8

TUBERCULOSIS IN OTHER SPECIES OF WILDLIFE

Abstract

To gain an understanding of the potential role of wild pigs, cats, stoats, goats, sheep, rabbits, hares, rats and mice in the dynamics of *Mycobacterium bovis* infection in free-ranging animals, numbers of these species from around Castlepoint and elsewhere in New Zealand, were examined for evidence of infection. The diagnosis of tuberculosis in most of the animals, (or parts thereof), was based upon gross necropsy followed by culture of suspect lesions, except in the case of cats and stoats, from which pooled lymph nodes were submitted for culture in each case. The findings were interpreted in the light of historical accounts of *M. bovis* infection in these species.

Thirty-three pigs were examined, with 28 of these from the Castlepoint area. Tuberculosis was diagnosed in 28 of the pigs, with the prevalence of tuberculosis in the Castlepoint pigs found to be 96%. The disease was generalised in 63% of 24 pigs in which sufficient of the carcass was available for examination. Pigs as young as 2 months showed severe disease. Suppurating sinuses were found in four animals. Although infection was thought to be acquired by ingestion of dead tuberculous animals, the results also suggested the potential for pseudo-vertical transmission, excretion from draining sinuses and respiratory tract in generalised cases, and from the pharynx in animals shedding from the tonsils. The possibility of significant intraspecific transmission was evident from the necropsy findings.

Of 62 cats examined, six (9.7%) were found to be infected with *M. bovis*. Four of these showed no gross tuberculous lesions. None of 19 juveniles examined showed evidence of infection. The results suggest that in the endemic areas cats become infected by consumption of tuberculous carrion, and that there will be a pool of infected animals without gross lesions. Despite having similar food habits to ferrets, cats from the same area showed a much lower prevalence of *M. bovis* infection, which suggests that the cat is more resistant to tuberculosis than the ferret, and unlikely to have a significant role in the epidemiology of tuberculosis.

Fifty stoats were necropsied, with ten collected from the Wairarapa, and the remainder coming from the North Canterbury area. Only three of the 40 (7.5%) stoats coming from North Canterbury were found to be infected, with none showing gross lesions. Infection is likely to have arisen from scavenging, especially by males, which appear to be more frequently infected. Compared with the prevalence

in ferrets from the same areas, the prevalence found in stoats was much lower, which taken together with the low occurrence of gross lesions, suggests that stoats are quite resistant to infection with *M. bovis*. Stoats are thus likely to be unimportant in the epidemiology of tuberculosis in New Zealand wildlife.

From the Castlepoint longitudinal study site, two of 13 (15%) sheep were found to be infected with *M. bovis*. Infection is thought to be acquired by contact with moribund tuberculous possums. Disease generalisation appears to be common. There is the potential for *M. bovis* to be moved to other areas free of tuberculosis, following unrestricted sheep transport. Although the possibility of transmission to cattle is slight, the potential role of sheep in upsetting domestic animal tuberculosis eradication programmes requires clarification.

In the Castlepoint environs, five of 37 (13.5%) free-ranging goats were found to have *M. bovis* infection. Of the five infected animals only one was found without gross lesions. Lesions in the remainder were of a minor nature, which suggests that the *M. bovis* strains in the Castlepoint area are not particularly pathogenic for goats. By analogy with other ruminants, infection is believed to be acquired by investigation of tuberculous possums. Wild goats appear likely to have an inconsequential role in the epidemiology of tuberculosis in New Zealand.

None of six hares, 61 rabbits, 45 ship rats and four brown rats were found to be tuberculous. Despite small numbers of hares, brown rats and one wild rabbit having been found infected with *M. bovis* elsewhere, it is believed that these species can be safely disregarded in tuberculosis control programmes.

Of the species investigated, only the pig appears to have sufficient potential for intraspecific transmission to be of concern in tuberculosis control programmes. However, evidence from overseas would suggest that they can usually be safely disregarded, as the prevalence of tuberculosis in these hosts has been found to fall in line with a reduction in the abundance of tuberculous carrion. Cats, stoats, sheep and goats appear to be simply spillover hosts, which may have a limited role in amplifying disease in the environment following possible, but limited, intraspecific transmission, and subsequent to their carcasses becoming available to scavengers. Rodents and lagomorphs are most unlikely to play any substantial role in the epidemiology of tuberculosis in New Zealand.

Introduction

Little is known of the contribution which mammals, apart from possums, deer and ferrets, make to the epidemiology of bovine tuberculosis in New Zealand. In an attempt to further the understanding of the potential role of pigs, cats, stoats, goats, sheep, rabbits, hares, rats and mice in the dynamics of the disease, these species were also examined for the presence of infection. As there was already a large body of data from possums in the Castlepoint area, as many other mammals as possible from the same area were examined for evidence of infection. With the less common species, such as pigs, stoats and cats, additional animals from tuberculosis endemic areas from further afield were also examined.

The environment around the Castlepoint study site is a mosaic of improved and poor quality pastures, scrub regrowth, pine plantations and native bush set amongst low coastal hills. This landscape provides a varied habitat which provides adequate cover and food for a wide variety of introduced mammals. Wild red deer (*Cervus elaphus*) and goats (*Capra hircus*) are common, and wild pigs (*Sus scrofa*), although not abundant, also range over the area. Although seen regularly, the low population of rabbits and hares supported few predators, such as ferrets (*Mustela putorius furo*), cats (*Felis catus*) and stoats (*Mustela erminea*). Weasels (*Mustela nivalis*), although present are rare. Rodents can be locally common, but are usually not present in high density.

The aim of this portion of the research was to draw together what was known of bovine tuberculosis in the range of mammals studied, and given the findings in these mammals around Castlepoint and elsewhere in New Zealand, draw conclusions as to their role in the epidemiology of *M. bovis* infection. It was particularly important to establish whether any other mammals, apart from possums, were potential reservoir hosts of tuberculosis, or likely to be important amplifying hosts of disease (Morris and Pfeiffer, 1995).

Materials and Methods

The necropsy technique used for all predators was similar to that employed in the examination of ferrets (described in Chapter 3), and involved the collection of pooled lymph nodes from all animals necropsied. The technique used in the small ruminants and pigs was similar to that described for deer in Appendix I, except that samples of tissue pools were not kept for mycobacteriological examination.

Tissues removed for bacteriology were stored in sterile plastic containers, and together with swabs and other samples, stored at -84°C. Samples were later submitted to the AgResearch Tuberculosis Laboratory, Wallaceville, for culture. Routine culture techniques described by Buddle *et al.* (1994) were used to isolate *M. bovis* from tissue samples. Urine and faeces were cultured using the method described by Jackson *et al.*, (1995b). Swabs were cultured by vortexing them in 10 ml of sterile-distilled water and then 5 ml of this solution was decontaminated by the addition of an equal volume of 0.75% w/v of cetyl pyridinium chloride. The remainder of the culture procedure for the swabs was the same as that used for tissues. Where appropriate, 95% confidence limits have been presented with results.

Pigs

Thirty three pigs were examined for the presence of *M. bovis* infection, with all but one of these being shot from the wild. Twenty eight of the pigs came from the Castlepoint environs and nearby properties, and the remaining five came from the Hauhungaroa Ranges in the central North Island endemic area. Whole carcasses of 15 pigs were examined by detailed necropsy. For the other pigs only portions of carcasses were available for examination, and included thoracic and abdominal viscera from two pigs, the head, thoracic and abdominal viscera from three animals, head, thoracic viscera and body of one pig, head and thoracic viscera from four animals, the head and abdominal viscera from one pig and the head only, from seven animals. Eleven pigs, or portions of pigs were stored frozen prior to examination. Age determination of 23 pigs was carried out using teeth eruption tables. Samples for culture were submitted from 30 pigs, and selected tissues from 18 animals were subjected to histopathological examination.

Cats

Sixty two cats were gathered from five areas on both the North and South Island, through 1994 and 1995. All were acquired by trapping, and kept chilled or frozen prior to examination. Necropsies and data recording similar to that conducted on the ferrets, were carried out on all cats, but with the omission of excretion site sample collection. Pooled tissue samples were retained from 55 of the cats, and these included portions of the retropharyngeal, respiratory and jejunal nodes. Only two lesioned cats, examined in early 1994, were subjected to histopathological examination.

Stoats

Stoats trapped in the Wairarapa (10) and North Canterbury (40) areas between December 1993 and January 1996 were necropsied following chilled or frozen storage, in the majority of cases. Samples from each animal included the complete pair of retropharyngeal nodes, jejunal node and two of the respiratory lymph nodes, which were pooled for mycobacterial culture. No histopathology was performed on any tissues from the stoats. Stomach contents were examined, and where food was present the contents itemised and a visual estimate made of the proportion of each food type, and the approximate volume of the stomach contents.

Sheep

Thirteen feral Romney sheep were mustered from the study site in Backdrop paddock between November 1993 and April 1994. These animals were shorn and subjected to tuberculin skin testing, using 0.1 ml of bovine tuberculin (tuberculin PPD [Bovine], 1 mg/ml, Central Animal Health Laboratory, Wallaceville Animal Research Centre) injected intradermally into the bare skin of the medial aspect of the thigh (Cordes *et al.*, 1981). All sheep were transported back to Massey University, where they were euthanased and necropsied on the third day following the tuberculin skin tests. Histopathology was performed on selected tissues.

Goats

Between April 1994 and February 1995, 37 goats shot on the two properties immediately surrounding the Castlepoint study site were examined. Seven of the goats were domestic Angora castrates, and the remainder were wild/feral mostly of the Angora type. The whole body including all viscera were available for detailed necropsy from eight animals, the head, body and thoracic viscera from 19, the head

and thoracic viscera from nine, and the thoracic viscera only, from one animal. Twenty two of the carcasses were frozen prior to examination. Tissues from 13 animals were submitted for mycobacterial culture, and 3 tissues were examined histologically.

Hares and Rabbits

Rabbits (*Oryctolagus cuniculus*) and hares (*Lepus europaeus occidentalis*) were shot or picked up as road casualties on properties surrounding the Castlepoint study site. Most were frozen prior to necropsy. Briefly, the necropsy procedure involved stripping the skin off the ventral body from the chin to the inguinal area. Peripheral lymph nodes, including the popliteal, axillary, retropharyngeal and mandibular were examined initially. The thoracic contents were inspected by palpating the lungs and examining the draining nodes, the mesenteric hepatic/gastric lymph nodes were incised and the liver, kidney and spleen inspected. Substantial care was necessary in the examinations, as the lymphatic tissue in rabbits and hares are small by comparison to their body size. A record was kept of the location from which the rabbits came, their sex, maturity, and physiologic status. Any suspicious lesions were retained for mycobacteriology, and histopathology.

Rats and Mice

Ship rats (*Rattus rattus*), brown rats (*Rattus norvegicus*) and house mice (*mus musculus*) are all found in the Castlepoint area, but are never abundant. Rats and mice were sometimes caught in leg-hold traps set for possums, or on other occasions they were caught in back-breaker traps baited with peanut butter or bacon and set specifically to catch rodents. A large number of back-breaker traps were set during the termination of the longitudinal study. All captured rodents were necropsied, and examined carefully for gross lesions. Samples of suspicious gross lesions were retained for mycobacterial culture, but no tissues were examined histopathologically.

Results

Pigs

The sample consisted of 16 females, which included four aged sows, six mature sows and six weaners. There were also fourteen male pigs, which included six mature animals, three yearlings, and five weaners. In a further three pigs the sex was unknown.

Gross lesions suggestive of tuberculosis were seen in 27 of the 33 pigs. Of the six pigs without lesions, one was an 8 month old farmed pig from Castlepoint, reared out of doors, and was likely to have had access to tuberculous carrion when in the custody of the previous owner. This animal was skin test positive prior to slaughter, and *M. bovis* was isolated from pooled head nodes. Two other non-lesioned animals also had tissues submitted for culture, but *M. bovis* was not isolated. Of the other three without gross lesions, two 10 month old animals came from a farm distant from Castlepoint, and the third pig only had the head available for examination, from which the jaw had been removed, and along with it most of the head-associated lymph nodes, thereby significantly reducing the chances of detecting lesions.

Mycobacterium bovis was isolated from 26 of 30 pigs which had samples submitted for culture. A further two pigs were also believed to be diseased, as they had gross lesions and histopathology consistent with infection, but no AFB visible in lesions. Acid-fast bacilli were visible in the lesions of only three of the 18 pigs subject to histopathology, however another 12 of these animals possessed granulomatous lesions consistent with a diagnosis of *M. bovis* infection. Tuberculosis thus appeared to be present in 28 of the 32 pigs examined, and from which a satisfactory chance of making a successful diagnosis was possible. Four of five (80%) animals from the Hauhungaroa Range, and 24 of the 25 pigs (prevalence $0.88 < 0.96 < 1.0$) from close to the Castlepoint study site, provided evidence of infection.

There was sufficient of the carcass and viscera examined in 24 pigs, to be able to tell if the disease had progressed beyond the alimentary tract and its associated lymph nodes. Generalisation of disease was identified in 15 (63%) of the 24 pigs, in which thoracic and/or peripheral node lesions were present.

The youngest infected pig was thought to be 2 months old, and showed lesions restricted to the head and alimentary tract. Four weaners thought to be 3 months of age, and another of approximately 4 months, were also infected.

All pigs in which lesions were present or in which *M. bovis* was isolated, had lesions in sites associated with the alimentary tract, such as head-associated lymph nodes, oropharyngeal tonsil, gastric (Figure 8-1), hepatic and mesenteric lymph nodes. Evidence of tonsillar infection, although not sought in all pigs, was found in ten animals where gross or microscopic lesions consistent with tuberculosis were visible. Gross tonsillar lesions identified included mucosal ulceration in 2 cases (Figure 8-2), 5 to 10 mm focal areas of raised mucosal surface over inflamed crypt tissue, variable-sized areas of submucosal caseation (Figure 8-2). Histologically most lesions appeared as small granulomas, with caseation and fibrosis occasionally being seen. Acid-fast bacilli were rare and seen in only two cases. Lesions in nodal sites varied from large draining mandibular abscesses (3 cases in mature animals) (Figure 8-3), and the more typical enlarged node with caseation, extensive fibrosis and mineralisation (Figure 8-4), through to barely detectable foci with caseation and calcification, often without noticeable fibrous reaction. Acid-fast bacilli were absent or rare in lesions subjected to histopathology. One notable case in an animal 34 months old, involving widespread and florid lesions, had a tuberculous femoro-tibial joint. This area was grossly enlarged, with a diameter of 15 cm, and contained extensive fibrous reaction surrounding multiple foci of caseous exudate, which was draining through the skin in several places.

Tuberculous thoracic lesions were seen in eight of 23 animals in which the lungs or thorax were examined. Severe thoracic lesions were only found in three animals, less than 8 months old. The most severe case seen involved multiple spherical lesions, up to 4 cm in diameter which bulged from both the collapsed lung surface and parietal pleura (Figure 8-5). Only the largest of the lesions contained necrotic centres. The lung lesions of older pigs were usually small calcified nodules, often only 2 or 3 mm in diameter. Calcified and fibrotic lesions in the respiratory lymph nodes were present in all cases, even in the apparent absence of pulmonary parenchymatous lesions in two animals.



Figure 8-1. Grossly enlarged tuberculous gastric lymph nodes (arrow head) in the lesser curvature of a wild pig's stomach.

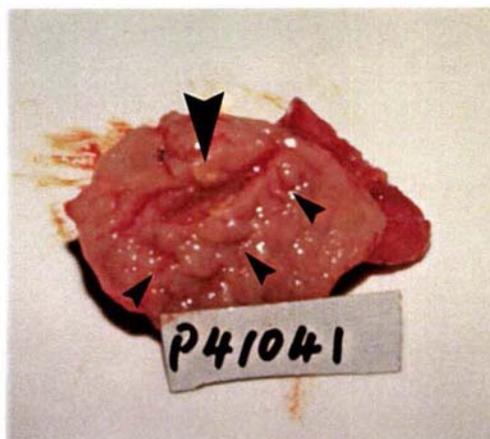


Figure 8-2. Excised soft palate of a tuberculous wild pig showing ulcerated surface (small arrow heads) and caseous tonsillar tissue underlying the incised mucosa (large arrow head).



Figure 8-3. Head of wild pig showing two tuberculous mandibular abscesses, one of which is draining its caseous contents.



Figure 8-4. Enlarged mandibular lymph nodes of a wild pig, incised to show extensive fibrous and caseous reaction to infection with *M. bovis*.

Lesions caused by *Metastrongylus elongatus* were present in eleven lungs examined, and were principally found in the dorso-caudal diaphragmatic lobes. Parasites were not always visible in or near the lesions. Several lungs contained nodular areas up to 1 cm in diameter, which were grey and homogeneous on incision and needed differentiation from tuberculous granulomas. Histologically these were identified as areas of lymphofollicular hyperplasia, and appeared to be associated with the presence (or prior presence) of pulmonary nematodes.

Florid and generalised lesions were common in all age groups. Excretion site sampling was attempted in 4 cases with widespread lesions. In two 4 month old animals with especially florid lesions, *M. bovis* was isolated from both tracheal and pharyngeal swabs. Culture of nasal cavity swabs, faeces, and urine from one, produced no evidence of excretion by these routes. Two other pigs, one aged female, and an 8 month old female, were also similarly sampled. Although both had apparent tuberculous tonsillar lesions and focal lung lesions, up to 2 mm in diameter, neither furnished isolates of *M. bovis* from pharyngeal, tracheal, nor nasal swabs, or from faeces.

Cats

There were 31 males (including 9 juveniles) and 31 females (including 10 juveniles) in the sample examined. Nineteen came from the Scargill and Tiromoana ferret control site in North Canterbury (Caley, 1995a). Sixteen came from the southern end of Lake Wairarapa, 17 from the Tinui/Castlepoint area, eight from near Featherston and two from near Carterton. *Mycobacterium bovis* was isolated from six (9.7%) of the cats. A brief description of each culture positive case appears below:

Case 1, a well conditioned mature female with multiple 2-3 mm caseous foci in a mandibular lymph node (Figure 8-6) from which *M. bovis* was isolated. Miliary lesions up to 3 mm were also scattered throughout the pulmonary parenchyma. A cranial mediastinal lymph node was also enlarged. This animal had tracheo-bronchial lavage fluid, faeces and a pharyngeal swab submitted for culture. No isolates of *M. bovis* were recovered from these samples.

Case 2, a mature male in average condition, whose only significant lesion was a slightly enlarged right caudal cervical lymph node, which contained several pale

streaky foci in the cortex. Part of the caudal cervical lesion was included with the pooled nodes.

Case 3, an aged female in good condition, with several 2-3mm cream coloured lesions in the diaphragmatic lung lobes. The lung lesions were thought to be parasitic in origin.

Case 4, an aged male in poor condition, with generalised lymph node enlargement, but without focal lesions. The right diaphragmatic lung lobe showed pleural fibrosis on the dorsal surface, and all major airways contained copious quantities of mucus.

Case 5, a well conditioned mature male with a parasitic gastritis and a heavy burden of ascarids. No tuberculous lesions visible.

Case 6, a mature female in average condition free of significant gross lesions.

Suspicious gross lesions were observed in another six cats. Two of these cases had pulmonary lesions, one of which was subjected to histopathological examination. The extensive caseous pulmonary nodules examined were found to be parasitic in origin, possibly caused by *Aelurostrongylus* spp. The other cat had similar but less extensive lesions, also attributed to parasites. Enlarged mesenteric lymph nodes were noted in three cats, one of which was subjected to histopathological examination, with no significant lesions being found. One other cat had a small calcified nodule in the ileo-caecal lymph node which was also possibly parasitic in origin.

For estimating tuberculosis prevalence, the cats were divided into two categories i.e. those derived from areas with high (Lake Wairarapa, Castlepoint, and Carterton), or low (North Canterbury and Featherston) prevalence of tuberculosis in local possums. None of the 27 cats, (0.0 - 0.03) from the low possum prevalence areas were found to be infected, whereas six of 37, (0.04<0.16<0.28) from the high possum prevalence areas were infected with *M. bovis*. The difference in tuberculosis prevalence between the two source categories was significant (Fisher's exact 2-tailed test, $p = 0.035$). The relative risk for those cats living in the high possum prevalence area was 43.9 (2.1 - 1281.2) compared to those in areas with few tuberculous possums.

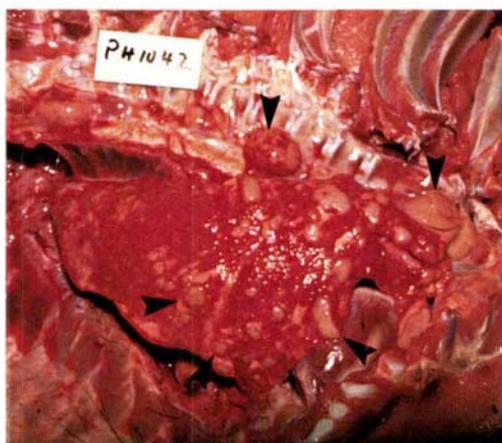


Figure 8-5. Lungs and parietal pleura of a young wild pig showing multiple large cream-coloured tubercles (arrow heads).



Figure 8-6. Head of a wild cat showing enlarged tuberculous mandibular lymph node containing foci of necrosis.

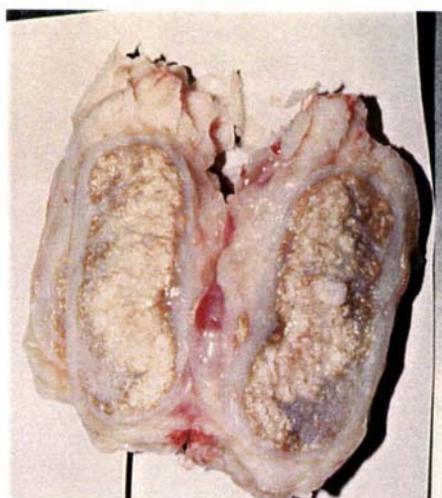


Figure 8-7. Right caudal cervical lymph node (8 by 5 cm) from the aged ewe. The lesion characterised by extensive fibrosis, calcification and necrotic foci.

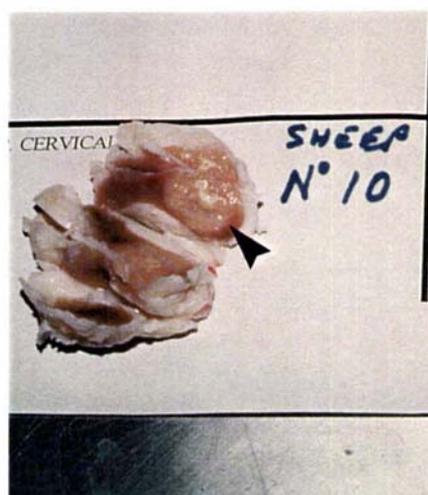


Figure 8-8. Left caudal cervical lymph node from the same sheep. The node is slightly enlarged and the tubercle (arrow head) is restricted to one pole only.

None of the 19 (0.0 - 0.04) juvenile animals had tuberculosis, whereas six of 43 (0.04<0.14<0.24) adult cats were infected. The age difference in prevalence however was not significantly different (Fisher's exact 2-tailed test, $p = 0.165$). The relative risk for mature animals was 26.6 (1.3 - 755.9) compared with juveniles.

Food items were identified in the stomachs of 32 cats. Evidence of carrion consumption was found in twelve stomachs (37.5%). Items categorised as carrion included: possum, putrid bird, sheep, foetal membranes and maggots. Possum remains were found in four stomachs (12.5%). There was no significant difference in the prevalence of carrion consumption between males and females ($\chi^2_1 = 0.08$, $p = 0.784$).

Stoats

All of the 40 stoats from North Canterbury came from either the Tiromoana or Scargill Landcare Research study sites (Caley, 1995a). Eight stoats came from around Castlepoint, but did not include any taken from the longitudinal study site of tuberculosis in possums. One other stoat was found dead several kilometres west of Castlepoint, and another was trapped near Featherston. Twenty-five of the stoats were females and included six recognisable as juveniles by their small size. Of the remaining 25 males, three were juveniles. Four of the immature animals, all weighing approximately 160 g were all from the same litter, collected as road casualties near Castlepoint.

None of the stoats had gross tuberculous lesions. Culture of pooled nodes produced isolates of *M. bovis* from 3 stoats, one of which was noted to have had an enlarged popliteal lymph node. Three other stoats were noted to have had lymph node enlargement of the retropharyngeal, jejunal and multiple sites, respectively, but were all negative on culture. All three infected animals were mature males from North Canterbury. The apparent prevalence of infection from the North Canterbury site was 0.075 (0.0 - 0.16), and this was much lower than the prevalence of 0.25 (0.18 - 0.33) seen in 137 ferrets examined through the same period. None of the Castlepoint stoats were infected, whereas nearly all ferrets taken from this area have been tuberculous.

The stomachs of 18 stoats contained food. In five cases this was entirely bait meat used as a trap lure. Rabbit, the most common item, was found in the stomach of

seven animals. Three other stomachs, two from males and one from a female, contained carrion, and this was fly-blown in two cases.

Sheep

All of the sheep were examined between November 1993, and April 1994. Of the 13 sheep mustered, there were six 2 year-old sheep including 5 castrates, and one ewe. There were three 15 month-old sheep including one ram, one castrate and two females. There was also one aged ewe with two 6 month-old lambs at foot. Three sheep reacted to the intradermal tuberculin test. These were the aged ewe, one 15 month-old female, and the 15 month-old ram. Of these three, only the ram and the old ewe showed gross tuberculous lesions at necropsy, and from both of these, *M. bovis* was isolated from tissue samples. The apparent prevalence of confirmed infection was 0.15 (0.0 - 0.35). A description of lesions, and other significant findings in the three tuberculin skin test reactors is set out below.

Case 1 was the aged ewe which was the only sheep which was believed to have been on the study site for a period of over 1 year. The skin test reaction was large and oedematous. This ewe had multiple lesions in numerous sites. The thoracic cavity showed lesions in the lungs, aortic, and all respiratory lymph nodes. The lymph nodes were enlarged, calcified and encapsulated by a thick wall of fibrous tissue (Figure 8-9). The lungs were adherent to the right chest wall, and contained numerous tubercles up to 4 mm in diameter scattered throughout the parenchyma and subpleural areas. Histologically, these granulomatous lesions contained a thick wall of epithelioid and giant cells surrounding areas of caseous debris. In the head, all lymph nodes of the right side were affected by tuberculous lesions, but none were found on the left side. The mandibular node was only 4 mm in diameter, calcified and adherent to the skin, suggesting that the contents of a caseous tubercle may have discharged through a sinus and had since healed. Both the right (Figure 8-7) and left (Figure 8-8) caudal cervical lymph nodes were tuberculous, with the right being grossly enlarged and heavily calcified.

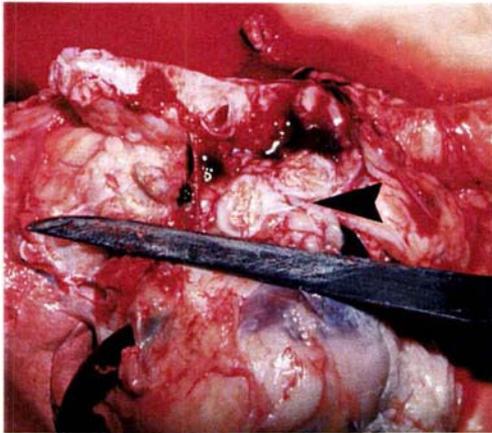


Figure 8-9. Right tracheobronchial lymph node from the aged ewe (arrow head). The node is enlarged and contains a central core of caseo-calcareous debris within a thick fibrous capsule.

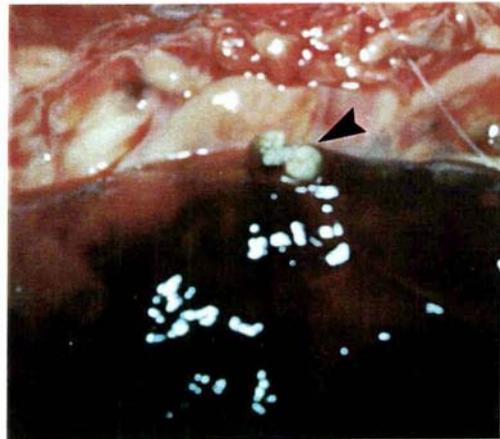


Figure 8-10. Small subpleural lung tubercle of a wild goat, incised to show caseous contents held within a thin fibrous capsule.



Figure 8-11. Severed head of a wild goat, showing tuberculous parotid lymph node with a caseous core surrounded by fibrous capsule.

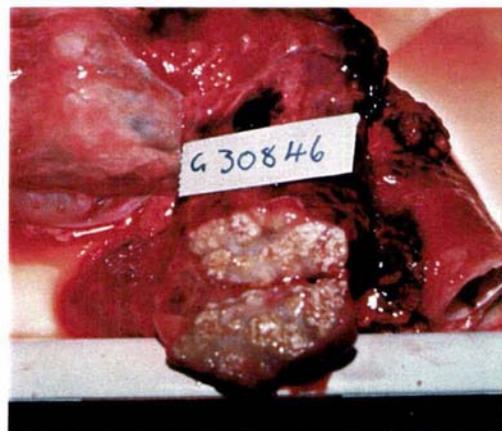


Figure 8-12. Tuberculous cranial mediastinal lymph node of a wild goat, showing enlargement, fibrosis and pockets of caseation necrosis.

Histologically the nodal lesions were composed of large coalescing granulomas, filled with necrotic debris, with large areas of mineralisation, surrounded by epithelioid and giant cells. A small calcified lesion was also found in a section of the jejunal lymph node. Isolates of *M. bovis* were recovered from lymphatic tissue, but not from swabs of the pharynx, and tracheal sputum, nor from faeces, urine and milk. No AFB were visible in tissue sections. The disease appeared to be quiescent at the time of necropsy, but must have been severe and florid at some time in the past, lesion regression being hampered by extensive fibrosis and calcification.

Case 2, a 15 month-old male, also showed a large oedematous reaction at the site of tuberculin injection. The only significant lesion was found in the right superficial cervical node, which was enlarged to one and a half times its normal size and contained a calcified fibrotic lesion. Histologically the node was found to contain numerous caseous and mineralised foci, and small numbers of giant cells in granulomatous areas, one of which was seen to contain a single AFB.

Case 3, a 15 month-old ewe showed a firm, 1cm raised lump in reaction to the tuberculin injection. No tuberculous lesions were found, but a pool of tissues including subpleural olive-green focal lesions, a portion of congested pneumonic lung, and sections of a mandibular and ischiatic lymph nodes, was sent for mycobacterial culture. *Mycobacterium bovis* was not isolated from the pooled tissues. Histological examination of samples from tissues pooled for culture showed that the focal lung lesions, which had also been observed in other sheep, were lymphoid nodular responses to nematode infection, and that the lymph nodes submitted were hyperplastic.

Goats

The estimated age, and sex distribution of goats examined is outlined in Table 8-I.

Table 8-I. Estimated age and sex distribution of necropsied goats. Number infected with *M. bovis* shown in brackets

Sex	Age		
	weaner	yearling	mature
castrate ^a			7(2)
female	3	8(1)	10(1)
male	3	3	2(1)

a five domestic and 2 feral

Mycobacterium bovis was isolated from five of the 37 (prevalence $0.025 < 0.135 < 0.245$) goats necropsied. Two of these had what were believed to be typical lesions at necropsy, another two had suspicious caseous lesions, and the fifth isolate came from a pulmonary lesion which was thought to be parasitic in origin. The individual cases are described briefly below.

Case 1 was a mature domestic Angora castrate. One of a group of five necropsied, which had become feral and had been free-ranging for several years. The dorsal diaphragmatic lobes contained multiple spongy spherical areas which collapsed poorly on opening the thorax. On incision these areas contained small irregular foci of olive-green colour. Histologically these lesions were typical of verminous pneumonia, with parasites and eggs in alveoli and airways, peribronchial smooth muscle hypertrophy, lymphoid hyperplasia, and occasional foreign body granulomas. Although the respiratory lymph nodes had a greenish discolouration there were no significant lesions found elsewhere in the goat. The isolate of *M. bovis* came from one of these verminous pulmonary lesions which was submitted for culture before their aetiology had been determined. Verminous pulmonary lesions, similar to those described above were found in 27 of the 37 (73.0%) goats examined.

Case 2 was a mature feral castrate of the Angora type. Only the head, forequarter and thoracic contents were examined. Apart from the parasitic changes in the thoracic viscera described in Case 1, the only significant lesion was in the left caudal cervical lymph node. This node showed a 5 mm diameter caseous focus surrounded by a thin fibrous capsule. At necropsy this lesion was thought suspicious of tuberculosis only. *Mycobacterium bovis* was recovered from this lesion.

Case 3 was a female of approximately 12 months age, from which the head, forequarter and thoracic contents were examined. The only significant lesion was found in the right caudal cervical lymph node. This node was enlarged to twice its normal size, and contained multiple 2 to 3 mm caseous tubercles scattered throughout the node.

Case 4 was a mature female wild coloured goat, in which the whole body, head and thoracic contents were examined. Lesions consisted of a 3 mm caseous lesion on

the pleural surface (Figure 8-10), an enlarged parotid lymph node containing a 2 cm cream-coloured caseous focal lesion surrounded by a fibrous capsule (Figure 8-11). The lungs also showed the typical diffuse verminous pneumonia described earlier, as well as several 2 mm diameter olive-green subpleural foci, with the appearance of lymphoid hyperplastic tissue. *Mycobacterium bovis* was recovered from a tissue pool composed of the caseous lung lesion and parotid node.

Case 5 was a mature male goat in which the whole body, minus the abdominal viscera. Significant lesions were confined to the thoracic cavity, where three pulmonary tubercles were found, ranging in size from 1 to 5 cm. The largest tubercle was associated with adhesions to the parietal pleura in the cranial thorax. Lesions were also seen in the caudal and cranial mediastinal lymph nodes (Figure 8-12) and in the cranial tracheobronchial lymph node. All tuberculous lesions appeared similar, and were caseous in nature with a small amount of calcification and extensive fibrous encapsulation. *Mycobacterium bovis* was isolated from a tissue pool composed of the caudal mediastinal lymph node and one lung lesion.

Four other goats were found to have suspicious lesions, but, which following culture, were found to be free of tuberculosis. In these animals one had a caseous oropharyngeal tonsil crypt, two had focal pulmonary lesions and enlarged respiratory lymph nodes (probably parasitic), and one other had multiple caseous head lymph nodes and a 5 cm diameter lamellated pulmonary caseous lesion (probably caseous lymphadenitis). Other lesions submitted for culture were respiratory lymph nodes and apparently verminous pulmonary lesions, all of which failed to provide isolates of *M. bovis*.

Hares and Rabbits

Six hares were examined, four from “Waio”, the same property as the study site, and two from a neighbouring farm. The sample comprised 4 adult females and 2 adult males, none of which showed any significant lesions. No samples were submitted for mycobacterial culture.

Sixty one rabbits were examined, 22 from the same property as the study site, and 39 from two adjacent properties. The sample was comprised of 27 females, which included three juveniles, and 34 males, which included nine juveniles. Suspicious lesions were found in three rabbits, and samples of each were submitted for

mycobacteriology. No isolates of *M. bovis* were recovered from the samples. The suspicious lesions included: one 2 mm cream-coloured focal liver lesion; and two 1 cm diameter caseous lung nodules from two rabbits. One of the lung nodules was examined histologically but the lesion bore no resemblance to a tubercle, contained no AFB, and was associated with local emphysema and bronchiectasis.

Rats and Mice

Through the period extending from February 1994 till April 1995, there were 45 ship rats, 4 brown rats and 14 mice examined.

The ship rat sample comprised 22 mature females, 21 mature males and two juvenile males. Thirteen of the ship rats came from the study site, four from elsewhere on the same property, 23 from the neighbouring property to the north of the study site, and another five from elsewhere in the district. Three of the rats showed gross lesions. One animal had a mesenteric lymph node three times larger than normal, one had approximately 20 grey foci up to 1.5 mm diameter over the surface of most lung lobes, and another had 7 pale focal lesions up to 1 mm diameter in the intermediate and diaphragmatic lung lobes. From each of these three ship rats lesions were submitted for culture, but no isolates of *M. bovis* were recovered.

The four brown rats, all mature females, were caught near farm outbuildings in the area. Two from one site showed multiple grey focal pulmonary lesions up to 1 cm diameter. The larger of these lesions contained caseous or mucoid material in the centre. No mycobacteria were isolated from any of these lesions.

Of the mice, seven were mature females and seven were mature males. Twelve were caught on the study site, and the other two from elsewhere on the property. One mouse showed multiple 0.5 to 1 mm grey focal lesions scattered over the lung surface. However, culture of the lung failed to isolate *M. bovis*.

Discussion

Pigs

There have been no published reports of *M. bovis* occurring at such a high prevalence (up to 96%) in feral pigs as has been found in this study. Earlier New Zealand reports from Central Otago have found the prevalence of histologically diagnosed infection to be 0.16 (0.12 - 0.21) (Wakelin and Churchman, 1991) and 0.32 (0.22 - 0.41) (Knowles, 1994), thus suggesting that there are significant differences in the epidemiology between the disease in Central Otago and the two tuberculosis endemic areas investigated in this study. The scavenging behaviour of pigs (Thomson and Challies, 1988), and the common occurrence of disease in other species, such as possums, ferrets, deer, wild goats etc. in both the Castlepoint and Hauhungaroa environs will ensure that 100% of pigs are likely to be exposed to the organism at some time, whereas the availability of tuberculous carrion in the Central Otago area is considered to be much lower. This suggests that those animals in this study not showing lesions are likely to have undergone lesion regression/resolution (Francis, 1958), and those lesions failing to provide isolates of *M. bovis* presumably having become free of AFB (or nearly so). Some animals with limited exposure to *M. bovis* may not develop significant lesions, yet still harbour the organism in lymph nodes. An example of such a case was the domestic pig without gross lesions, but from which *M. bovis* was isolated. Isolation of the organism is common from lesion-free sites (Feldman, 1936; Ray *et al.*, 1972).

The high prevalence of generalised disease (63%) suggests that many of the animals became infected at an early age, when disease progression is likely to be more severe. This was typified by all young pigs being infected, the youngest being 2 months of age. These showed the most severe pulmonary lesions of any of the animals. This data suggests the possibility of pseudo-vertical transmission occurring in these young pigs. Such transmission from mammary lesions has been noted in domestic pigs by M'Fadyean (1915, cited by Francis, 1958) and Albiston *et al.*, (1954), and was the possible cause of infection in piglets, approximately 1 month of age examined in a cross-sectional study in Central Otago (Knowles, 1994).

The occurrence of bacillary excretion via draining sinuses, from the mouth, and from the respiratory tract (as noted in this series and also by Plum and Slyngborg,

(1938)), also suggests that apart from the potential for offspring of sows to become infected, there will be some risk to companions of generalised cases, where contact is sufficient and rate of excretion high. Infection of pen mates by other pigs with generalised disease has been demonstrated experimentally by Ray *et al.*, (1972).

The range of cases examined here is not large enough to draw inferences about the routes of infection and their relative frequency. This study does however support the oropharyngeal tonsil as a route of bacillary entry (Chaussé, 1915, cited by Francis 1958), and possible excretion site for *M. bovis* infection in pigs. Chaussé (1915) described early tonsillar lesions as small mucosal elevations centred around crypt entrances, followed later by caseation, ulceration in some cases, and eventually resolution and scarring. In this series ulceration of the tongue was also noted, and it is likely that this is an additional manifestation of *M. bovis* infection, despite no AFB being visible in the lesions, as lingual ulceration is the most common manifestation of oral tuberculosis in humans (Çelik *et al.*, 1995).

The results suggest a possible role for pseudo-vertical transmission, and excretion from suppurating sinuses, from the respiratory tract in active generalised cases, and from the pharynx in animals shedding from the tonsils. Observations in other studies in New Zealand and in Australia (Comer *et al.*, 1981; Wakelin and Churchman, 1991; Knowles, 1994; McInerney *et al.*, 1995) have failed to show a high prevalence of generalised disease, and this is likely related to the probability of contact with the organism and size of the infecting inoculum ingested. The pigs from Central Otago are unlikely to eat tuberculous carrion frequently, as infected wildlife is scarce in this environment (Ragg *et al.*, 1995b). The moderate to low historical prevalence observed in the feral pigs of the Northern Territory despite apparent ready access to tuberculous buffalo (*Bubalis bubalis*) and wild cattle carcasses (Letts, 1964; Comer *et al.*, 1981) could possibly have arisen through only more resistant older juveniles and mature pigs having access to these carcasses at the end of the dry season, and the bacillary load in these carcasses being low. The high temperatures generated during putrefaction in these large animals in a tropical environment may be sufficient to kill the low numbers of mycobacteria present before scavenger ingestion.

The most useful site to examine for diagnostic purposes is the head, as lesions are most prevalent in the head-associated lymph nodes. The soft palate should also

always be examined for tonsillar disease. Culture of pooled head nodes and a portion of the soft palatine tonsillar tissue should suffice to allow isolation of *M. bovis* from infected animals showing no gross lesions.

The disease was found to be more severe in younger pigs, and although dramatic and severe lesions were present in several animals, published reports (Francis 1958) would suggest that these animals may not necessarily have died, as lesions may resolve over time in porcine cases. Acquisition of infection seems to occur principally from the ingestion of possum, and other tuberculous carrion. Feral pigs will thus provide a means of identifying, on a broad scale, the areas in which tuberculous wildlife can be found. The gregarious nature of pigs, and the large litters produced (McIlroy, 1990) suggest that it would be unwise to underrate the potential for either pseudo-vertical or horizontal transmission to occur. However despite these transmission mechanisms being available, it would seem that once the load of *M. bovis* in the environment is reduced, such as has occurred in the Northern Territory of Australia (McInerney, *et al.*, 1995), the number of generalised cases, and hence potential bacillary excretors will fall, thereby ensuring that the feral pig does not maintain the disease in the absence of other infected hosts.

Cats

Gross lesions readily attributable to tuberculosis were seen in only two of the six cats from which *M. bovis* was isolated. As with other species there appears to be a pool of animals which are infected with *M. bovis* but which do not possess gross lesions, either because they had not progressed to that point or because lesions had regressed and healed without eliminating infection from the body (Snider, 1971). The comparatively low prevalence of disease (16%) in cats from areas with prevalent possum tuberculosis, is in sharp contrast to that of ferrets in the same environment, in which up to 100% can be infected. Cats clearly scavenge a good deal and (like ferrets) will eat dead tuberculous possums when available (Fitzgerald and Karl, 1979; Langham, 1990). The apparent difference in prevalence between ferrets and cats in the same environment probably results principally from the higher susceptibility of ferrets to disease after an oral challenge, although differences in the interval from detectable infection to death may also contribute. Cats will kill and eat stoats (Fitzgerald and Karl, 1979; Wodzicki, 1950) and although not recorded, they could also eat or fight with ferrets. It is thus possible

that cats could become infected through contact with mustelids. The prevalence of disease in cats with typical gross lesions in this study (5.4% in the high prevalence area), is similar to historical accounts, where cats fed contaminated milk or meat, usually had prevalences of infection between 2 and 10% (Snider, 1971). The prevalence of gross lesions is also comparable to previous New Zealand studies where figures of 2.0, 2.0 and 4.2, 0.9, 0 and 1.3% prevalence have been reported by Walker *et al.* (1993), Cowan (1994), Ragg *et al.* (1995b), Caley (1995a) and Oliver (1996) respectively.

Only in the studies reported by Cowan (1994), have the tuberculous lesion sites been described. The three cats apparently had one lesion each, and these were found in the mesenteric and mandibular lymph nodes, and lung. It is likely that cats which develop tuberculous pulmonary lesions or generalised disease, may die after a short clinical course, of perhaps 40 to 50 days (Francis, 1958), and so be less likely to be represented in cross-sectional surveys. This may partly explain the observed difference (approximately one order of magnitude) in prevalence between cats and ferrets from the same environment, as infected ferrets are likely to survive for at least six months after infection (Dunkin *et al.*, 1929; Symmers *et al.*, 1953). The apparent scarcity of cats with multiple lesions in these wildlife studies may also be explained by rapid disease progression and death in animals with multiple lesions.

The apparent importance of tonsillar and retropharyngeal lymph node lesions in other species which are commonly infected by the oral route is not apparent in the cat. Lesions of the retropharyngeal lymph nodes of cats are not commonly reported in the literature (Francis, 1958), whereas those of the mandibular and mesenteric lymph nodes occur commonly. The mandibular lesions may arise as a consequence of oral mucosal injury whilst consuming tuberculous prey, or alternatively could indicate that some of the efferent drainage from the oropharyngeal tonsils may pass to these nodes. The low frequency of retropharyngeal involvement may simply reflect that although subject to infection, this node is not a predilection site for lesion development. It is recommended for future mycobacteriological studies of cat populations, that the mandibular, caudal cervical and ileo-caecal lymph nodes be included in pooled tissue samples, together with the respiratory and retropharyngeal

nodes, as this should improve the chance of successful culture from cats without gross lesions.

Despite the high susceptibility by the respiratory route, and the multitude of potential excretion sites i.e. oral, respiratory, urinary, venereal, congenital, and via open skin lesions (Francis, 1958), the cat “is not an easy victim to the disease” (Jennings, 1949). The alimentary tract in feral cats appears likely to be the major route of infection, and cats seem moderately resistant to this mode of transmission. As with ferrets, there appears to be a broad scale association between the occurrence of tuberculosis in feral cats and the presence of tuberculosis in domestic stock. The limited New Zealand studies to date have however shown the prevalence of disease in feral cats to be considerably lower than ferrets under comparable circumstances, despite both species sharing similar dietary components, and in comparable proportions. Cats are most unlikely to be reservoir hosts of the disease in New Zealand, as bovine tuberculosis has been successfully eradicated in many areas where, historically, there has been a moderate prevalence of disease in cats associated with infection in cattle. Cats may however be capable of transmitting infection to stock, as has been demonstrated for possums and ferrets (Sauter and Morris, 1995a), but the chances of this occurring to any significant extent will be small. There have been no reports of moribund tuberculous cats seen abroad in daylight, and common sites for lesions are not well suited to extensive excretion of bacilli.

Stoats

The disease has not previously been recorded in animals without gross lesions, as in no other studies have non-lesioned animals had tissues subjected to culture. The 7.5% prevalence of tuberculosis in stoats from the North Canterbury area, is low in comparison with the prevalence of 25.5% identified in ferrets, using the same techniques, and sourced from the same area, at the same time.

Tuberculosis has only been identified in stoats from the South Island, with the disease first recorded by Coleman (1975) in animals trapped as a bycatch in a possum investigation in the Hohonu Ranges of the West Coast. No details were provided with these early cases, but since that time, a small number of additional infected stoats have been found.

Ragg *et al.* (1995b) found one female amongst 61 stoats collected in Central Otago with tuberculous lesions at necropsy and from which *M. bovis* was isolated. Gross lesions in this animal were found in the jejunal, right retropharyngeal and right caudal cervical lymph nodes (J. Ragg pers. comm.). Histologically the jejunal lymph node lesions were characterised by macrophage accumulation with areas of necrosis containing numerous AFB. Of 548 ferrets captured from the same area, 17.9% were found to be infected with tuberculosis. Coleman *et al.* (1994a) found two of two mature male stoats captured on the West Coast in 1992 infected with tuberculosis. Diagnosis was confirmed by culture of lesions, which in these animals consisted of a grossly enlarged jejunal lymph node (35 by 20 mm) in one, and a slightly enlarged jejunal node (15 by 8 mm) in the other (R. Jackson, pers. comm.). These cases came from an environment where the prevalence of tuberculosis in possums was exceptionally high, at approximately 60%. The following year (1993) a mature male, one of three stoats trapped in the same area was found to have a lesion in the jejunal lymph node from which *M. bovis* was isolated. The prevalence of disease amongst the possum population in 1993, was found to be 16.7%. The following year (1994), at the same site, neither of a further two stoats examined showed evidence of tuberculous lesions (Coleman and Cooke, 1995). The apparent prevalence of tuberculosis in the possums at this time was lower at 7.8%.

Other studies where predators have been examined and failed to find tuberculous stoats include the report of Walker *et al.* (1993), in which one necropsy-negative stoat was examined from an area in which 15.5% of 84 ferrets were found to be infected. In a series of five cross-sectional studies reported by Cowan (1994), nine stoats were captured, but none were suspected of being tuberculous at necropsy. Three of these stoats (one each), came from three areas where tuberculosis had been found in ferrets, the prevalence ranging from 1.7% to 10.2%.

Six of seven infected stoats, for which details were recorded both in this and earlier studies, have been males. The lesion sites or the tissues from which *M. bovis* has been isolated suggests that ingestion of contaminated material was the likely source of infection. An explanation for this apparent difference in prevalence between the sexes is variation in the diet. Males are known to eat more possums and carrion than females (King and Moody, 1982). Murphy and Dowding (1994) found possum remains in 20% and 29%, of gut and scat samples respectively in males, with no

evidence of possum consumption detected in females. Similarly, possum was found in the gut content of 4 of 65 male stoats, but none of 31 females examined, and was also found in 21% of 33 scats from known males, but in none of 48 scats from females examined by Murphy and Dowding (1995). It is unlikely that possums are killed by male stoats, as they would be too formidable a prey, but Murphy and Dowding (1994) did find that male stoats were more likely to frequent roadsides than females, and also more likely to scavenge road-killed possums. Although three stomachs in the current study contained carrion, and two of 18 contained maggots, putrid carrion consumption has been reported to occur only rarely in studies where stoats living in forest country have been investigated (King and Moody, 1982; Murphy and Dowding, 1995), thus suggesting that stoats inhabiting farmland may be forced to eat more carrion.

Cannibalism is another possible means of infection in predators, however evidence for its occurrence was seen in only four of 1514 stoat gut samples examined by King and Moody (1982), and one nestling stoat has also been found in the gut of one of 65 male stoats examined by Murphy and Dowding (1995). This suggests that the likelihood of transmission by this means is low.

There is some indication that the disease is more likely to be identified in stoats living in areas with a high prevalence of tuberculosis in possums. Although a moderate prevalence of tuberculosis exists in the possum population around Castlepoint, no tuberculous stoats were identified from this area. This may have been because only three of the eight animals were mature, and of these three, only one was a male.

Despite the fact that stoats can become infected with *M. bovis* they are unlikely to develop gross lesions, and those lesions that do develop, being of minor significance, and likely to resolve. The absence of significant gross lesions also suggests that bacillary excretion will be poor, and the probability of transmission to members of their own species, or others, low. Thus stoats are unlikely to have a significant impact on the epidemiology of the disease in wildlife despite their potential for high reproductive rates and wide dispersal of offspring. Their low abundance in farming areas, and their small size will make them inconspicuous and unlikely to attract the attention of inquisitive stock even if sickening from *M. bovis* infection.

Sheep

Historically the reported prevalence in sheep has always been very low, and well under 1% (Francis, 1958). However, the findings of two New Zealand reports, detailing the disease in sheep grazing in endemic areas, suggest that the prevalence of disease was approximately 10 to 15% (Davidson *et al.*, 1981; Cordes *et al.*, 1981), which is comparable to the current findings. There has been little published on the investigation of tuberculosis in sheep in New Zealand, probably due to the adverse agro-political ramifications, but occasional cases of tuberculosis are reported (Black and Orr, 1996; J. Adams, and G. Pannett, pers. comm.).

Sheep are believed to be as susceptible to *M. bovis* infection as cattle (Francis, 1958; Wilson and Miles, 1975), and have on occasion been found to suffer severe outbreaks of tuberculosis (Uhl and Müller, 1995). The scarcity of disease reports in sheep may stem from the fact that sheep usually do not show clinical signs when infected (Carmichael, 1938a), and because the lesions can be easily mistaken for those of caseous lymphadenitis (Allen, 1988). The widespread, but quiescent lesions in the aged ewe of this study, are testimonial to the severe generalised disease which can occur and which has previously been reported in New Zealand (Davidson *et al.*, 1981; Cordes *et al.*, 1981) and Germany (Uhl and Müller, 1995).

The lesion distribution in sheep suggests that both the respiratory and oral routes of infection are important in this species (Davidson *et al.*, 1981; Cordes *et al.*, 1981; Uhl and Müller, 1995). An interesting observation in the sheep from this study, that of Cordes *et al.* (1981), and Uhl and Müller, (1995), was that of the body nodes showing lesions, only those of the head, and the caudal cervical nodes were involved. This suggests that the caudal cervical nodes are predilection sites for haematogenously disseminated infection, which may be analogous to the high, but unexplained, prevalence of disease found in the axillary nodes of possums (Jackson *et al.*, 1995a).

There was evidence of a mandibular lymph node having discharged in the aged ewe necropsied. Cordes *et al.*, (1981), were able to isolate *M. bovis* from 3 of 10 bronchial swabs cultured from sheep with pulmonary lesions. This suggests that there is potential for both transmission between sheep, and between sheep and other species, given the right circumstances. However, in this study it is not believed that infected sheep became so through contact with cohorts, but rather through direct

contact with terminal tuberculous possums. In a series of trials where livestock were faced with sedated possums placed on the pasture (so as simulate terminally tuberculous animals), sheep generally showed little interest (Sauter and Morris, 1995a). However, despite the level of interest being much lower than that shown by cattle and deer, some sheep were shown to make brief contact with the possums. Contrary to the situation in cattle and deer, the level of interest in possums increased with habituation to possum exposure. This suggests that, as with other livestock that some sheep may have infectious contact with possums, but this is likely to be at a much lower frequency than for cattle and deer under similar circumstances. This direct contact could readily explain the high frequency of apparently orally acquired infections in New Zealand sheep. The high proportion of generalised cases resulting from a period of prolonged infection in a susceptible host.

The current evidence suggests that although sheep can suffer severe disease following infection with *M. bovis*, that the disease process may be self limiting, and un-associated with significant horizontal transmission amongst flock mates. There is some concern however, that substantial numbers of sheep in endemic areas may become infected through the investigation of moribund tuberculous possums, and that the free movement of sheep to other areas could potentially provide a source of infection for other domestic stock in those areas. Although the possibility of transmission to cattle appears slight, the potential role of sheep upsetting domestic animal tuberculosis eradication programmes requires clarification.

Goats

This is the first time that the actual prevalence of tuberculosis in a group of wild goats in New Zealand has been reported. The moderate prevalence (13.5%) found in these goats is comparable with the findings reported by Sanson (1988), who found that in wild goats captured from the endemic areas on the West Coast that on average 7.2% were skin test positive. In some areas up to 31% of goats were positive to the skin test, and of those which were necropsied 45.5% showed lesions. Although reports of tuberculosis in goats in New Zealand are few (Allen, 1987; Sanson, 1988), there have been no tuberculous wild goats found from areas without *M. bovis* infection present in sympatric possum populations. As is suspected with wild deer, most transmission of infection is probably direct from terminal tuberculous possums cases to inquisitive goats. Historically, reports of tuberculosis

in goats have recorded disease in only a few animals, with prevalences below 2% (Francis, 1958). As goats are fully susceptible to *M. bovis* infection (Griffith, 1907), this suggests that intraspecific transmission is uncommon, despite the occasional occurrence of mammary lesions and infection of suckling offspring (Francis 1958). However, recent reports from Spain would suggest that there are strains of *M. bovis* which are goat specific in the Mediterranean countries (Gutiérrez *et al.*, 1995). Spanish goat herds suffer high prevalence infections, and up to 50% annual mortalities as a consequence (Vidal *et al.*, 1995; O'Reilly and Daborn, 1995).

The lesions found in the goats around Castlepoint were similar to those seen in cattle, and to those described by Sanson (1988). However, calcification was not a feature, as it often is with bovine tuberculosis, and some nodal lesions resembled those seen in deer. No goats were found with severe lesions, but the occurrence of single site lesions in the caudal cervical nodes suggests generalisation via haematogenous spread, and non-progressive disease.

Verminous pneumonia was an extremely common finding in the goats. Most of the lesions were believed to have been caused by *Dictyocaulus filaria*, which commonly infects domestic goats, and causes large florid lesions in the caudo-dorsal lung lobes (Valero *et al.*, 1992). The isolation of *M. bovis* from one such lesion was surprising. However, this was not thought to have been due to sample contamination as there were no other animals with tuberculosis necropsied on the same day, and the REA type was of a strain found in a variety of species, including possums, and principally restricted to animals taken from the same locality in which the goats had been running. This may have been, yet again, another example of an isolate coming from a non-lesioned site in an animal with non-progressive/regressed disease. Cousins *et al.* (1993), has also recovered *M. bovis* from a non-lesioned respiratory lymph node of a tuberculin skin test positive goat in Australia.

The currently available data suggests that goats in New Zealand suffer from a self-limited disease process, presumably after acquiring infection from possums. They are likely to be simple spillover hosts with no significant role in the epidemiology of tuberculosis.

Hares and Rabbits

The sample of hares was too small to draw any conclusions about the likelihood of their being any infected in the Castlepoint area. However, in localities where the disease has been/is endemic, including New Zealand, tuberculous hares have occasionally been found (Paine and Martinaglia 1928 (cited by Keet *et al.*, 1996); Schliesser, 1985; de Kantor *et al.*, 1984 (cited by O'Reilly and Daborn, 1995); Cooke *et al.*, 1993; Coleman *et al.*, 1994b). This suggests that if a large enough sample size had been collected that infected hares may have been found in the study area. The apparent low prevalence of infection in hares living in areas in which the disease is endemic, suggests that hares are far more susceptible to oral infection than rabbits, or that there is some aspect of their behaviour, or interaction with other hosts, which predisposes them to infection. Because of the low population densities of hares, and limited contact with other hosts, this species is likely to be of little importance in the epidemiology of tuberculosis in New Zealand.

The absence of tuberculosis in the rabbits was not unexpected as the disease has only ever been found in one wild rabbit (Gill and Jackson, 1993), despite evidence of infection being sought in many thousands examined in New Zealand. This one case was thought to have arisen as a result of a bite wound from an infected predator. Although the rabbit is extremely susceptible to airborne transmission of tuberculosis (Francis, 1958), they are apparently quite resistant to oral infection. This suggests that they may be too timid to approach other infected species closely enough to be at risk of airborne infection, and that if their feed or water is contaminated that they may be able to successfully avoid the development of disease. The finding of only one infected wild rabbit to date, strongly suggests that rabbits will play no significant role in the epidemiology of the disease in New Zealand.

Rats and mice

As few mice were necropsied in this study, and samples cultured from one only, little can be said regarding their infection status. Mice are considered to be innately highly resistant to mycobacterial infection (Francis, 1958), and there have been no instances of natural infection of mice reported. It is unlikely that mice will have any significant role in the epidemiology of disease in wild animal populations.

Rats are highly resistant to disease following infection with *M. bovis*. Experimental inoculations have shown that granulomatous pulmonary reactions follow infection. Lesions may be visible as pin-head sized foci in the lungs, which are typically composed of lymphocytic infiltrates and activated macrophages (epithelioid cells), which appear to be highly protective (Wessels, 1941; Ratcliffe and Palladino, 1952; Thorns *et al.*, 1982). However, small numbers of wild brown rats in Europe have been naturally infected with *M. bovis* (Bosworth, 1940; Vöhringer, 1964; Little *et al.*, 1982). These cases have arisen in the presence of infection in other species, such as cattle, pigs and badgers, with the rats thought to have been infected through eating excreta infected with *M. bovis*, or through access to infected offals.

Although no infected rats were found in the current study, only a few samples from lesioned animals were cultured. As most infected rats are likely to show no lesions, a larger study in which samples from all necropsied rats are submitted for culture will be necessary to establish whether ship or brown rats infected with *M. bovis* exist in New Zealand. In areas with endemic tuberculosis, a low prevalence of infection in wild brown rats may occur through consumption of tuberculous carcasses of other hosts. If rats are infected they may provide another source of infection for predators, but because of their well demonstrated resistance to infection, they could never be considered as candidates for reservoir host status, and thus must have an unimportant role in the epidemiology of tuberculosis.

Summary

Of the species investigated, only the pig, with its multiple potential routes of transmission, and involvement of juveniles, would seem potentially capable of being classified as a reservoir host. However, this may be an artefact of the tremendous exposure to *M. bovis*, which this species must have in endemic areas, where the prevalence of disease is moderate to high in many other mammals. Given the high exposure of cats and stoats to tuberculous carcasses, and the low prevalence of tuberculosis in these species by comparison with ferrets, they could only be classified as spillover hosts, with a minor role in the amplification of disease in the environment. Goats, although susceptible, are likely to play an unimportant role in the epidemiology of tuberculosis in wildlife, whereas sheep, which are more likely to develop florid lesions, are potentially capable of limited horizontal transmission. Sheep transport may pose a hazard to the eradication of

disease in non-endemic areas, but this aspect of the epidemiology of tuberculosis requires further investigation. Infection of lagomorphs and rodents, wherever it may occur, is likely to be inconsequential to the epidemiology of tuberculosis in other wildlife.

Acknowledgments

I am indebted to Ron Goile and the local field staff at Castlepoint, including Donna Lewis, and others who provided so many of the specimens for examination. For the submission of necropsy specimens by others of the Wairarapa farming community, I am also indeed grateful. Peter Caley (Landcare Research) provided a large number of cats and stoats collected from his study sites, and Graham Nugent (Landcare Research) also provided a number of pigs from the Hauhungaroa Range for examination. Mycobacterial culturing was performed by Geoff de Lisle and Gary Yates AgResearch, Wallaceville. Financial support from the Animal Health Board is gratefully acknowledged.

All of the necropsies on the above species were conducted by the PhD candidate. Data collection, storage, and subsequent analyses have also been carried out by the candidate.

CHAPTER 9

THE DIAGNOSIS OF BOVINE TUBERCULOSIS IN POSSUMS: TESTS AND THEIR APPLICATION

Abstract

Three indirect ELISAs, a culture filtrate, an MPB70 and a monoclonal antibody blocking assay, were evaluated, using naturalistic and pseudo-retrospective sampling, as diagnostic tests for tuberculosis in possums. A lymphocyte transformation assay was also evaluated using naturalistic sampling. The methodology employed logistic regression modelling and established cutoffs with the help of receiver operating characteristic curves. Infected possums were identified by culture of *M. bovis* or the presence of acid-fast bacilli in characteristic granulomatous lesions. The number of gross and gross plus microscopic lesioned organ sites were determined and used as an indicator of the stage of the disease in individual possums.

All ELISAs had low sensitivity when a cutoff selected to maximise the specificity was chosen. None of the ELISAs reliably detected possums infected with tuberculosis and they therefore have limited value for epidemiological studies. Although the culture filtrate and MPB70 ELISAs appeared to support an association between advanced disease states and humoral immune responses, the monoclonal antibody blocking assay performed with equal efficiency across all stages of the disease. Pseudo-retrospective sampling gave results which were generally comparable to those achieved with naturalistic sampling.

The lymphocyte transformation assay appeared to have a sensitivity of approximately 80%, when the specificity was set at 99%. A positive test response appeared to be driven by the presence of significant amounts of *M. bovis* antigen. This assay was the best of those evaluated, with the moderate sensitivity allowing it to be used with a high degree of confidence to retrospectively diagnose disease, and aid the development of hypotheses regarding the epidemiology of tuberculosis in possums.

The tests were applied to sera and blood samples from possums from the Castlepoint longitudinal study of tuberculosis in possums. The monoclonal antibody blocking ELISA was applied to 1477 sera, the MPB70 ELISA was used on 1281 sera, the culture filtrate assay on 310 sera, and the lymphocyte transformation assay was used on 745 blood samples. The results of ELISA and lymphocyte transformation testing allowed the identification of another 79 apparently infected possums, in addition to the 109 previously found by culture, histopathology, clinical

examination and necropsy, from amongst the population of 979 which had been trapped and examined on and around the site.

The additional data arising from these assays suggested that perhaps as few as one fifth of study site possums which had contact with *M. bovis* had been previously detected as infected by clinical examination. Test positive animals may have exhibited resistance to the disease, and/or have had resolved lesions or cryptic infection. These animals could potentially be included among the pool of possums in which reactivation of tuberculosis in the future was possible. The time from earliest evidence of infection till death, in those possums which showed clinical disease, varied from months to several years.

Introduction

A reliable and simple blood test which could be used to detect tuberculosis in possums would be welcomed by researchers and disease control personnel alike. Epidemiological studies of tuberculosis in possums are hampered by the lack of a suitable test with an ability to reliably determine disease status and time of initial infection. Currently, infection status is usually only determined by culture of biopsy material from palpable lesions in live animals and by careful necropsy plus extensive histopathology and or cultural examination of a wide range of tissues in dead animals. Experienced prosectors are required for reliable necropsy examinations, and laboratory cultural procedures are time consuming and expensive.

In man and other species, serological tests which rely on detection of a humoral response have proved disappointing for diagnosis of tuberculosis. Agglutination, complement fixation, precipitation and anti-globulin tests have all been investigated but have given poor results. Because enzyme linked immunosorbent assay (ELISA) has an ability to detect very low levels of circulating antibody, this method has received recent attention and several ELISAs have been evaluated in cattle (Auer 1987; Fifis *et al.*, 1989; Wood *et al.*, 1992; Hernández de Anda *et al.*, 1996; Costello *et al.*, 1997). Unfortunately, all of the tests dependent on circulating antibody have had poor efficiency and this has precluded their use as primary tests, although there is a general perception that they may detect some cattle which are non-responsive to the intradermal test. This is based on the premise that circulating antibody may be more detectable in cases of advanced disease. An ELISA test developed for badgers has also similarly showed low efficiency (Goodger *et al.*, 1994; Clifton-Hadley *et al.*, 1995). Deer however, seem to have a higher level of humoral response to infection than other species so far investigated (Griffin *et al.*, 1991; Griffin *et al.*, 1994), which has allowed ELISA testing to be successfully employed in tuberculosis eradication programmes.

At one time the possum was thought to have a deficient cell-mediated immune system, as splenic lymphocytes were found to be less responsive to PHA stimulation than those of rabbits (Moriarty, 1973). However Ramadass (1980) showed that the immune functions of the possum, such as production of antibodies in response to injections of SRBC and bovine serum albumin, and blood leukocyte

mitogenic responses, were as efficient as those of other metatherians, and compared favourably with those of eutherians. Moriarty and Thomas (1983) carried out further experiments with possums, in which delayed-type hypersensitivity reactions to PPD, and lymphocyte transformation assay (LTA) responses of peripheral lymph nodes to PPD were compared with those of guinea pigs. The skin test responses and the lymphocyte transformation indices showed that the susceptibility of possums to tuberculosis was not due to any inability to respond to mycobacterial antigens.

Three ELISAs for tuberculosis in possums were recently evaluated (Buddle *et al.*, 1995) using sera from experimentally infected animals, sera from tuberculous possums caught during a study by Coleman *et al.* (1994) and sera from a presumed non-diseased possum population in Northland. In the study reported here, sera from a larger sample, which included those naturally infected and tested by Buddle *et al.* (1995), were used for evaluation. This report also extends the initial evaluation of the three indirect ELISA assays conducted by Jackson (1995), as the current study has benefited by the inclusion of additional sera.

From April 1989 till October 1994, a longitudinal study of tuberculosis in a free-living population of possums has been conducted on a 21 ha farmland site in the Wairarapa (Pfeiffer, 1994; Jackson, 1995). During this period possums were captured in cages during 3 to 5 day trapping periods each month. All trapped possums were clinically examined every two months, or less regularly if the animal was infrequently captured. During each examination an attempt was made to collect a blood sample via cardiac puncture into a plain blood tube for later serum extraction. From April 1991 onwards, a number of possums also periodically had heparinised blood samples taken and submitted for LTA. If suspicious lesions were found at examination, samples were collected for mycobacteriology in an attempt to confirm the infection status of the possum. The majority of animals which died or were found dead during the study were necropsied, with pooled nodes or lesions submitted for culture. During September and October 1994 (the termination), trapping was carried out weekly, and all captured possums on the study site and surrounding area were examined, bled, and euthanased. Each animal was subjected to detailed necropsy, with samples of tuberculous lesions, or pooled nodes from non-lesioned possums retained for culture from animals which had previously been

captured. A large number of serum samples collected between April 1989 and June 1992, and after May 1994 were subjected to ELISA testing to help determine infection status, and time of initial infection. The results of the ELISAs, and LTA are presented, along with cases of particular interest, and data of significance to the understanding of the epidemiology of tuberculosis in possums.

The reasons for this investigation were twofold. One purpose was to examine whether any tests, such as LTA or ELISAs for tuberculosis using blood samples collected in the field, may accurately diagnose the disease in cross-sectionally sampled populations. Such assays could complement the diagnostic procedures already employed. The second aim was to allow fuller investigation of the epidemiology of tuberculosis in possums which had been captured in the longitudinal study at Castlepoint. Through application of the evaluated tests to samples collected from the possums throughout the study, it was hoped to identify additional infected possums to those which had already been found by conventional means.

Materials and Methods

Necropsies and determination of infection status

All dead possums used in the test evaluation were subjected to an extensive necropsy examination as described by Coleman *et al.* (1994a), during which details on the origin, sex, reproductive status, estimated age, weight and length, were recorded. Lesions from diseased animals, and pools of major body nodes, which included the superficial and deep axillary, inguinal, superficial and deep cervical, mesenteric and bronchial lymph nodes, were collected from non-lesioned possums, placed into sterile containers, and stored frozen prior to submission to the AgResearch Tuberculosis Laboratory, Wallaceville for mycobacterial culture. A positive diagnosis was made if culture of lesions or pooled lymph nodes and/or extensive histopathological examination of multiple organs as described by Cooke *et al.* (1995) demonstrated the presence of *M. bovis* or acid-fast bacilli (AFB). Diagnoses of tuberculosis in live animals were based on palpation of enlarged superficial lymph nodes with subsequent confirmation by isolation of *M. bovis* from aspirates obtained from the affected nodes. Necropsies were designated 'gross' (as opposed to detailed) when no ancillary laboratory procedures were performed. Detailed necropsies were conducted on all possums in which visible lesions suggestive of tuberculosis were detected.

Samples for serology

Serum samples for determination of tuberculosis status, were collected on six separate occasions from three possum populations (Table 9-I). Bloods were taken from possums during a series of cross-sectional studies conducted during investigations of the pathogenesis and prevalence of tuberculosis in possums (Coleman *et al.*, 1994a; Jackson *et al.*, 1995a), and also from a longitudinal study of tuberculous possums at Castlepoint (Pfeiffer, 1994; Jackson, 1995). Another 100 sera from possums believed to be free of tuberculosis were derived from Northland, a non-endemic area for bovine tuberculosis infection.

Retrospective examination of data from the 5 years of the longitudinal study also revealed that additional sera, which had been subjected to ELISA testing, could be included in the evaluations. Assay results from uninfected possums were used if necropsy and culture of pooled lymph nodes taken within 30 days of the serum sample being collected, showed no evidence of infection. Results from infected

animals were used if tuberculosis was diagnosed by isolation of *M. bovis*, and it was certain that at the time of blood collection the animal was infected. The results from any particular possum from the longitudinal study were used only once in the assay evaluation data set.

Table 9-I. Origins of sera used for ELISA evaluation, including category of diagnostic criteria used

Study (date)	Total necropsies	Number with Tb	Detailed necropsies	Gross necropsy only
Waio (July 1993)	84	1	13	71
Waio (Sept 1993)	57	3	10	47
Flagstaff (Aug 1992)	59	34	45	14
Flagstaff (Aug 1993)	51	7	51	0
Castlepoint-longitud. (1989-1994)	44	34	44 ^a	0
Castlepoint-longitud. termination (1994)	233	22	218 ^b	0
Totals	528	101	381	132

a diagnostic samples often collected from live animals

b 14 animals previously tuberculin primed and hence ineligible for evaluation

On 8 August 1994, 20 possums from the longitudinal study site were given 0.1 ml of bovine tuberculin (Tuberculin PPD, 1 mg/ml, Central Animal Health Laboratory, Wallaceville Animal Research Centre.), by intradermal injection into the thick hairless skin of the left plantar aspect of the tarsus. Fourteen of these possums were recaptured 4 to 5 weeks later at the termination, and sera collected both at the time of tuberculin injection and death was subjected to CF and MPB70 ELISA testing. Results of the assays was examined with the use of a paired t-test. Sera from these animals were not used for evaluation of the ELISAs.

During the cross-sectional studies, blood samples from non-sedated leg-hold trapped animals were collected immediately after death into plain blood tubes, as the blood ran from the nasal cavity, following a crushing blow to the cranium. During the longitudinal study, cage-trapped possums were tranquillised with an intramuscular injection of ketamine hydrochloride (40 mg/kg) prior to intra-cardiac blood collection. At the termination of the longitudinal study possums were anaesthetised with a mixture of ketamine hydrochloride (40 mg/kg) and diazepam (2 mg/kg) given by intramuscular injection prior to intra-cardiac blood collection. Sera were removed from clotted bloods after centrifugation, and stored temporarily

at -20°C until they could be transported to Massey University, where they were then transferred to permanent storage at -80°C . Sera collected from the Northland possums, were temporarily stored at -20°C .

Serological tests

The sera were subjected to three tests i.e. two indirect ELISAs and one blocking ELISA. The indirect ELISAs were developed using *M. bovis* culture filtrate (CF) and MPB70, a purified protein from *M. bovis* culture supernatants. The blocking immunoassay measured the serological response to an individual MPB70 epitope of *M. bovis* by competitive inhibition of the binding of murine monoclonal antibody. All assays were conducted at the AgResearch Central Veterinary Laboratory at Wallaceville using the procedures described by Buddle *et al.* (1995).

Culture filtrate and MPB70 test results were expressed as “absorbance indices”, calculated by expressing each result from the test serum as a fraction of the binding of a high positive reference serum. Blocking assay results were calculated as percentage inhibition of binding of monoclonal antibody as compared with a negative reference serum, divided by 100.

Samples for lymphocyte transformation assay

During the longitudinal study heparinised blood samples were also periodically collected for examination by LTA. The bloods were collected through the period April 1991 to June 1993 (529 useable results), and again from June 1994 till the termination of the study (216 useable results), with the majority (146) of the previously trapped possums killed at the termination having blood submitted for LTA. Assays on purified leukocytes were performed in triplicate according to the technique described by (Buddle *et al.*, 1992). Three stimulation indices were obtained by dividing the mean counts per minute (cpm) for the lymphocyte cultures incubated with each of, bovine PPD, avian PPD, or Concanavalin A (Con A), by the mean counts per minute of the unstimulated control cells. Due to the lengthy period over which the LTA assays were performed, changes in equipment, and the heterogeneous nature of the test, a rigorous selection protocol was developed to ensure that only reliable avian and bovine tuberculin stimulated cell culture results were included in the test evaluation. Outliers were removed if the coefficient of variation was greater than 60%, and the data discarded altogether if the

unstimulated control was greater than 10^4 cpm, or the Con A response was less than 10^4 cpm, or if the stimulation index (control to Con A) was less than 10.0.

Lymphocyte transformation assay results employed for test evaluation came principally from amongst the 428 animals killed at the termination of the longitudinal study, but also included 45 samples collected during the longitudinal study. The evaluation data set included samples from 144 non-infected, and 23 infected possums. Repeated measures from 8 infected animals were used in the evaluation to increase the power of statistical tests. The evaluation was run with and without the repeated measures but their inclusion seemed justified given the low number of infected possums in the sample, and that their incorporation served to narrow confidence limits, and did not significantly affect the estimates of specificity or sensitivity.

Test evaluation

For evaluation of the ELISAs and the LTA, detailed necropsy, described above, with or without isolation of *M. bovis* was used as the "gold standard" against which the other tests were compared. Sera from Northland, the 1992 Flagstaff study, and 38 of the sera from the longitudinal study were used in the evaluation of the monoclonal antibody blocking (BLOCK) assay (n = 82). Sera from Northland, the 1992 Flagstaff study and cross-sectional studies after 1992 were assayed with both the CF (n = 337) and MPB70 ELISAs. These MPB70 ELISA samples were also supplemented by an additional 34 longitudinal study samples thereby providing 371 sera for evaluation. Because separately prepared conjugate was used for the latter series of tests (post 1992), results from that series were adjusted by the difference in the means of the negative sera, recorded with the different conjugates in the two assays. This necessitated subtracting 2.0 and 1.6 OD units from the latter CF and MPB70 ELISAs. Samples incorporated from the Castlepoint longitudinal study were selected without knowledge of the assay result, and persons who performed all assays were unaware of disease status of the animal from which the sera was derived. Because the CF and MPB70 absorbance indices were not normally distributed, they were \log_{10} transformed for all calculations and analyses which required normally distributed values.

Tests were evaluated using three methods to allow a comparison of the techniques to be done. The first method used pseudo-retrospective sampling (Kraemer, 1992),

in which the negative Northland sera were used to establish a cutoff point for the tests on the other populations sampled. The cutoff was calculated as the mean absorbance index or percentage binding inhibition, plus 2.57 standard deviations. This cutoff corresponds to the point which separates the top 0.5% of values and should theoretically give a cutoff value consistent with 0.995 specificity.

The second method involved naturalistic sampling (Kraemer, 1992) in which the detailed necropsy results were applied, in conjunction with receiver operating characteristic (ROC) curves (Figures 9-2 and 9-3) to establish a cutoff point. The cutoff point was taken as the index value which allowed the specificity to be kept above 0.98, and maximised the test positive likelihood ratio statistic.

The third method involved logistic regression analysis, whereby predictive equations for the probability of infection were derived from the test results and other measured parameters. Independent variables which were screened in the logistic models included; area of origin, disease prevalence, sex, maturity, condition index (CI) (see Appendix III), lactational status, adjusted MPB value, adjusted CF value, blocking assay result, bovine to control stimulation index (B/C SI) and the bovine to avian stimulation index (B/A SI). The resultant predictive equations were then used to generate a series of ROC curves derived from the test evaluation data sets, so as to allow another set of cutoffs for each regression model to be produced which once again maintained the specificity above 0.98, and maximised the test positive likelihood ratio statistic.

To assist with the multivariate test evaluations and with the application of the results, the point prevalence of infection during the longitudinal study was determined for each month (Figure 9-1). During the longitudinal study, the presence of possums which were found to be tuberculous was recorded. Diagnoses were based principally on culture results of sampled suspicious lesions in live animals, but a few were diagnosed on the basis of necropsy result, histological examination or clinical examination. Eighty nine possums were found to be tuberculous by these methods over the 67 months of the study. To obtain a realistic estimate of the point prevalence of infection on a monthly basis, the crude figures were adjusted to account for the period in which possums were infected, but not diagnosed as such by the examiners, either because the possum had not been trapped for a period, or because the disease produced no clinical signs. Possums

which died from the disease were deemed to have been tuberculous for the preceding 6 months prior to the one in which they died, unless it was known that they were diseased for a longer period. This 6 month period was selected as a conservative estimate of the average duration of infection prior to death, as Jackson (1995) has shown that from the time an infected possum is first captured, the average time to death is 6 months, and Pfeiffer (1994) found the average time to death after lesion detection was 2 months, a period which must be preceded by a time of subclinical infection of unknown duration. Animals which died through accident, or consequent to cardiac puncture, and in which tuberculosis was subsequently diagnosed were deemed to have been infected for a lesser period, set arbitrarily at 3 months prior to the month of diagnosis. Possums which were first diagnosed by clinical examination were also deemed to have been infected for 3 months prior to the month of diagnosis. The number of apparently infected possums calculated for each month was further adjusted upwards by adding an additional 25%, a value conservatively believed to represent the proportion of infected, but non-lesioned animals, in the population (derived from the necropsy and culture of pooled nodes or lesions of most possums at the termination of the longitudinal study, where 7 of 27 known to be infected showed no gross lesions). The denominator i.e. the population at risk, used to calculate the point prevalence was estimated with the use of Jolly-Seber mark-recapture analysis of possum capture data using the programme "Caro" (Pledger *et al.*, 1995) (see appendix II). Both crude and adjusted point prevalence estimates are presented in Figure 9-1.

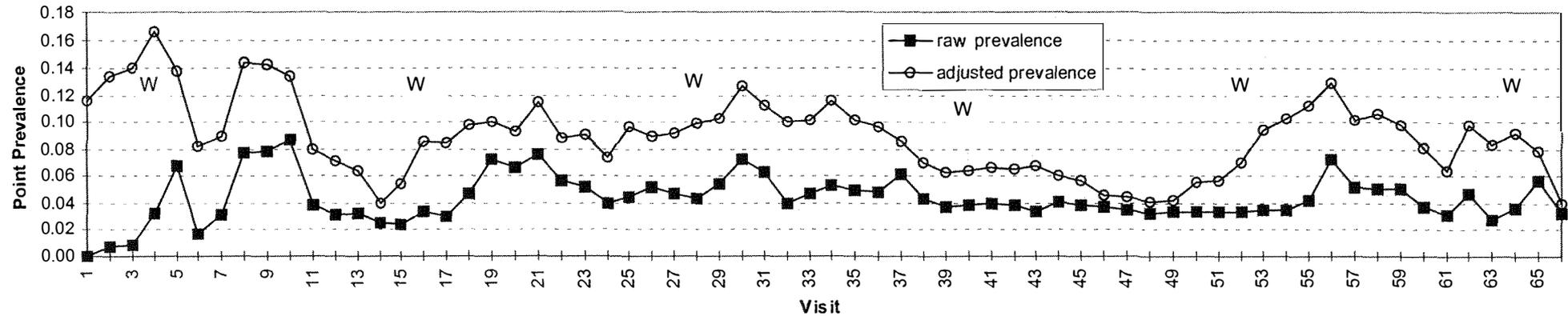


Figure 9-1. Plots of unadjusted, and adjusted estimates of point prevalence of tuberculosis in possums at the Castlepoint study site. “W” denotes the mid-winter month of July

Survival analysis

Kaplan-Meier survival curves, with pairwise logrank comparisons, were generated from data on possums in the longitudinal study (Figure 9-5), to examine whether a blood test positive status was associated with a reduction in life expectancy (months). The possum population was divided into 4 categories i.e. 1) those which were found tuberculous by either culture, histopathology, necropsy or clinical examination ($n = 110$); 2) those which had evidence of infection, but only from blood tests ($n = 78$); 3) animals believed to be non-infected and known to have died ($n = 536$); 4) the remainder of apparently non-infected animals which were lost to follow up (disappeared, $n = 255$). Entry into the data set was from the time of known birth, or estimated birth date. Time of failure was considered to be the time of known, or estimated time of death, or disappearance. If an animal was lost to follow up it was considered to have disappeared between the time of previous capture, and the following trapping episode at the study site. Possums dying or disappearing during a month were considered to have survived for that month. Right censoring occurred when a possum was known to have died accidentally (usually a casualty of cardiac puncture), or was deliberately killed or euthanased.

Survival analysis was performed on all possums showing a positive LTA response, and for which there was B/A SI data available. The last positive LTA result from 76 possums was used in the analysis, and the B/A SI value was stratified into high and low values, by splitting either side of the median value of 1.67. The survival of these test positive animals from the estimated date of birth, right censored by accidental death or killing was used to construct a Kaplan-Meier survival curve. The two resultant curves were compared on the basis of the logrank method. A subset of the possums which were positive to the LTA were categorised as either known infected, because *M. bovis* had been isolated at some time, or were classed as lost to follow up. Survival from date of birth, till time of death or disappearance of these two classes was investigated by constructing Kaplan-Meier survival curves, and comparing these using the logrank method as performed previously.

Other

An investigation into the relationship between the prevalence of gross lesions, and the prevalence of those with no gross lesions (NGL) which were investigated by culture of pooled lymph nodes or extensive histopathological examination, was carried out using the data from the termination, and the results of five applicable cross-sectional studies reported by Jackson *et al.*(1995a) and one study reported by Hennessy *et al.*(1986). The data were combined by a three way categorisation of disease prevalence based on the presence of gross lesions i.e. 1) 0 to 10%, 2) 11 to 20%, and 3) above 20%. Chi² for trend investigating the relationship between gross prevalence and prevalence of necropsy-negative possums was conducted.

Test evaluation statistics were generated in the spreadsheet software Microsoft Excel for Windows, version 5 (Microsoft Corporation, Redmond,WA.) using Testview, Version 1.05 (I. Gardner and J. Holmes, Department of Epidemiology and Preventative Medicine, School of Veterinary Medicine, University of California, Davis, CA.) and ROC curves were constructed using NCSS Version 6.0 (J. Hintze, Kaysville, Utah, USA). Agreement between tests was analysed with the aid of computer software, WinEpiscope 1.0 (N. de Blas, C. Ortega, K. Frankena, and J. Nordhuizen. Veterinary Faculty, Zaragoza, Spain, and Agricultural University, Wageningen, the Netherlands.). Jolly-Seber population estimates were produced with the aid of the software package Caro (S. Pledger and A. Tokeley, Victoria University of Wellington, and M. Efford, Landcare Research, Dunedin,

1995). Logistic regression, paired t-test, and survival analysis were performed using the software package SPSS, version 7 (SPSS Inc., Chicago, IL). Ranges in estimates presented are bounded by 95% confidence limits.

Results

Logistic regression

A variety of models were developed which could be used to predict the probability of infection being present at any one test episode, for possums from the longitudinal study, given a result of one or more tests on any particular occasion. The use of some independent variables in the models was constrained by the origin of the samples, or the total number of cases available. Area of origin was highly correlated with prevalence and so could not be used as a covariate. No first order interaction terms proved to be influential, nor were maturity, sex or lactational status found to have significant effects in any model. ELISA values were of no predictive value in models which included LTA results. Insufficient data was available to derive a regression model for the Blocking ELISA which incorporated significant terms. The final models which were applied to the blood tests from the longitudinal study possums are presented in Tables 9-II to 9-V.

Results of the logistic regression model incorporating the B/Cs, only, or the B/C and B/A SI proved to be very similar to the initial test evaluation based on simple ROC cutoffs. This was because the models failed to incorporate any other significant predictor variables, possibly as a result of the small sample size and the data coming from populations with little variation in prevalence of disease. Prevalence was however shown to have a significant effect on the outcome of models including the CF and MPB70 ELISAs. Condition index had predictive value in the MPB70 model only, where it was found that those in better condition were more likely to furnish a positive test. The addition of the CI and prevalence terms in the model still however made only marginal improvement to the usefulness of this test with the sensitivity from the ROC cutoff remaining below 15% (Table 9-VII), whereas the addition of prevalence to the CF model produced substantial improvement in the apparent sensitivity of this assay, increasing the sensitivity from 24% to 45%.

Table 9-II. Logistic regression model based upon the bovine/control stimulation index only (applied to LTA results of 17 longitudinal study possums for which the B/A value was unavailable)

Covariate	Coefficient	SE	DF	P-value
Constant	-9.73	2.73	1	< 0.001
B/C SI	0.63	0.18	1	< 0.001
Prevalence	0.76	0.36	1	0.037

n = 174, Deviance = 58.7, $\chi^2_2 = 107.4$, p < 0.001

Table 9-III. Logistic regression model based upon both the B/C and B/A ratios. Was applied to 728 LTA results of the longitudinal study possums

Covariate	Coefficient	SE	DF	P-value
Constant	-5.74	1.03	1	< 0.001
B/C SI	0.61	0.20	1	0.002
B/A SI	1.56	0.68	1	0.021

n = 174, Deviance = 57.7, $\chi^2_2 = 102.3$, p < 0.001

Table 9-IV. Logistic regression model based upon the Culture Filtrate ELISA evaluation. This model was applied to 310 test results of longitudinal study possums from the second half of 1994

Covariate	Coefficient	SE	DF	P-value
Constant	-3.97	0.40	1	< 0.001
Prevalence	0.06	0.01	1	< 0.001
CF	0.05	0.01	1	< 0.001

n = 332, Deviance = 221.4, $\chi^2_2 = 114.4$, p < 0.001

Table 9-V. Logistic regression model based on the MPB70 ELISA evaluation. The predictive model was applied to 1281 test results from the longitudinal study possums

Covariate	Coefficient	SE	DF	P-value
Constant	-5.78	1.18	1	< 0.001
Prevalence	0.06	0.01	1	< 0.001
CI	0.38	0.16	1	0.014
MPB	0.09	0.02	1	< 0.001

n = 365, Deviance = 287.1, $\chi^2_3 = 118.4$, p < 0.001

ROC analysis

The ROC curves of three LTA based tests are plotted in Figure 9-2, and the ROC curves of the three ELISAs applied to the possum data are shown in Figure 9-3. The lines show the relationship between the sensitivity and specificity as the value of one or the other is varied. The greater the area under each curve (Table 9-VI), the more discriminating is the test.

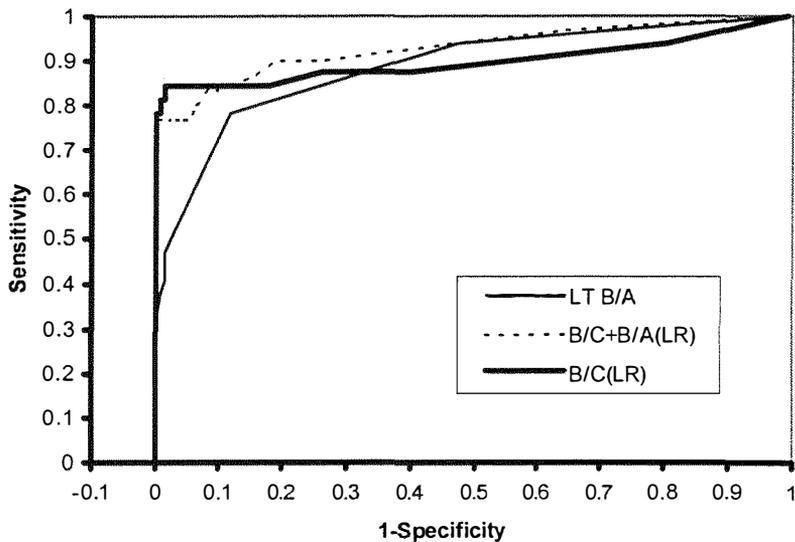


Figure 9-2. Receiver operating characteristic curves plotted for three lymphocyte transformation assay based tests. (LT B/A = bovine/avian SI; B/C(LR) = logistic regression model based on bovine/control SI only; B/C + B/A(LR) = logistic regression based model involving the two SIs)

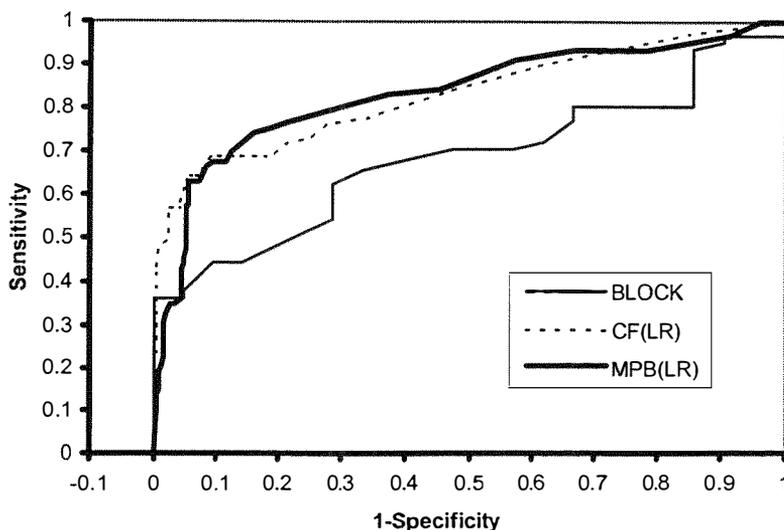


Figure 9-3. ROC curves plotted for the three ELISA based tests which were found to be the most useful (BLOCK = blocking ELISA, and MPB = MPB70 ELISA, and LR denotes that the test was based upon the results of the appropriate regression model)

Because of low sample numbers, and the absence of matching, the confidence intervals on the areas under these curves were wide, and consequently no test could be identified as being significantly better than another. However the LTA based tests appear to perform considerably better than the ELISA based tests. Of the two stimulation indices the B/C ratio appears to provide higher sensitivity when the specificity is close to 1.0. The blocking ELISA assay also shows higher sensitivity than the other two ELISA assays when the specificity is 1.0, but is inferior to the other ELISAs when the specificity is reduced. There is a suggestion from the area under the curves that the Blocking and CF ELISA assays may be more discriminating for males than females.

Table 9-VI. Area under the ROC curves for the various assays, and their subsets based on age and sex, used to diagnose tuberculosis infection in possums (additional details presented in Table 9-VII)

Assay	Area	95%CI	SE
CF all	0.726	0.50-0.95	0.111
CF ♂	0.777	0.49-1.00	0.143
CF ♀	0.644	0.29-0.99	0.175
CF m	0.725	0.48-0.97	0.123
CF j	0.741	0.22-1.00	0.261
CF ^{LR}	0.830	0.60-1.00	0.116
MPB70 all	0.645	0.46-0.83	0.091
MPB70 ♂	0.642	0.40-0.88	0.120
MPB70 ♀	0.655	0.38-0.94	0.140
MPB70 m	0.647	0.45-0.85	0.100
MPB70 j	0.636	0.18-1.00	0.229
MPB70 ^{LR}	0.833	0.64-1.00	0.101
Blocking all	0.690	0.49-0.89	0.101
Blocking ♂	0.723	0.39-1.00	0.139
Blocking ♀	0.664	0.39-0.94	0.136
Blocking m	0.674	0.46-0.88	0.105
Blocking j	0.828	0.28-1.00	0.276
LT B/A	0.886	0.55-1.00	0.170
LT B/C	0.882	0.54-1.00	0.170
LT B/A ♂	0.866	0.46-1.00	0.204
LT B/C ♂	0.887	0.49-1.00	0.200
LT B/A ♀	0.946	0.25-1.00	0.346
LT B/C ♀	0.859	0.23-1.00	0.317
LT B/C ^{LR}	0.898	0.56-1.00	0.171
LT B/C + B/A ^{LR}	0.928	0.58-1.00	0.178

♀ = females; ♂ = males; m = mature; j = juvenile; LR = from regression model

Sensitivity and specificity results of the various assays, and using the cutoffs shown are presented in Table 9-VII. The sensitivity of the three ELISA assays is poor. The Blocking assay has a higher sensitivity than the MPB70 test, when the ROC cutoffs are chosen. Although the sensitivity of all ELISAs is low, there are subgroups, for which the sensitivity appears to be improved. The CF and MPB70 tests seem to perform better with males and juveniles however the Blocking ELISA appears to be better with females and juveniles. The application of the ROC derived cutoffs to the Blocking and the CF assay made no difference to the cutoff chosen, whereas the ROC cutoff was substantially higher than that selected using the negative Northland sera in the MPB70 assay, which reduced the sensitivity of the assay substantially. The predictive logistic regression models produced slight sensitivity improvement for the MPB70 assay, but considerably improved the sensitivity of the CF assay from 0.24 to 0.45.

Table 9-VII. Summary of ELISA and LTA results, including subsets based on sex and age (ROC generated cutoffs, selected to maximise the likelihood ratio, and maintain specificity above 0.98, have been used except where indicated)

Assay	Cutoff	N	Prevalence	Efficiency	Sensitivity	Specificity	LR ⁺	χ^2_1
CF	1.83	337	0.20	0.84	0.24(0.15-0.36)	0.99(0.97-1.0)	32.2	56.8
CF ^{PR}	1.82	337	0.20	0.84	0.24(0.15-0.36)	0.99(0.97-1.0)	32.2	56.8
CF ^{LR}	0.64	336	0.20	0.89	0.45(0.33-0.57)	1.0(0.98-1.0)	120.4	126.3
CF♂	1.78	185	0.23	0.85	0.38(0.24-0.54)	0.99(0.95-1.0)	27.2	49.8
CF♀	1.89	152	0.16	0.85	0.12(0.03-0.32)	1.0(0.96-1.0)	>10.2	15.6
CFm	1.83	283	0.19	0.85	0.27(0.17-0.41)	0.99(0.97-1.0)	31.1	54.7
CFj	1.54	54	0.22	0.85	0.42(0.17-0.71)	0.98(0.86-1.0)	17.5	14.6
MPB70	1.69	371	0.25	0.78	0.12(0.07-0.21)	1.0(0.98-1.0)	33.9	30.2
MPB70 ^{PR}	1.27	371	0.25	0.81	0.26(0.18-0.37)	0.99(0.96-1.0)	18.5	61.2
MPB70 ^{LR}	0.86	365	0.24	0.79	0.14(0.08-0.23)	1.0(0.98-1.0)	37.2	33.7
MPB70♂	1.35	201	0.26	0.80	0.25(0.15-0.40)	0.99(0.96-1.0)	37.3	35.2
MPB70♀	1.71	170	0.23	0.79	0.10(0.03-0.25)	0.99(0.95-1.0)	13.4	9.5
MPBm	1.69	313	0.25	0.78	0.13(0.07-0.23)	1.0(0.97-1.0)	30.7	27
MPBj	1.05	58	0.24	0.85	0.36(0.14-0.64)	1.0(0.9-1.0)	>15.7	17.2
Blocking	0.22	82	0.74	0.52	0.36(0.25-0.50)	1.0(0.81-1.0)	>7.9	10.4
Blocking ^{PR}	0.22	82	0.74	0.52	0.36(0.25-0.50)	1.0(0.81-1.0)	>7.9	10.4
Blocking♂	0.2	43	0.74	0.47	0.28(0.25-0.50)	1.0(0.68-1.0)	>3.8	3.9
Blocking♀	0.22	39	0.74	0.67	0.55(0.36-0.73)	1.0(0.66-1.0)	>5.5	9.4
Blocking m	0.23	70	0.76	0.56	0.42(0.28-0.77)	1.0(0.77-1.0)	>7.1	10.3
Blocking j	0.96	12	0.83	0.67	0.75(0.36-0.96)	1.0(0.40-1.0)	>3.0	6
LT B/C	4.24	176	0.18	0.96	0.78(0.60-0.90)	0.99(0.95-0.99)	112.5	124.7
LT B/C♂	4.25	103	0.22	0.94	0.78(0.56-0.92)	0.99(0.92-1.0)	62.6	70.4
LT B/C♀	4.58	72	0.13	0.97	0.78(0.40-0.96)	1.00(0.93-1.0)	>16.3	54.3
LT B/A	2.66	174	0.18	0.88	0.41(0.24-0.59)	0.99(0.96-1.0)	48.0	44.7
LT B/A♂	1.73	102	0.22	0.87	0.45(0.25-0.67)	0.99(0.92-1.0)	36.4	35.0
LT B/A♀	1.56	71	0.11	0.94	0.63(0.26-0.90)	0.98(0.90-1.0)	39.4	34.0
LT B/C ^{LR}	0.31	176	0.18	0.96	0.81(0.63-0.92)	0.99(0.96-1.0)	117.0	130.8
LT B/C-B/A ^{LR}	0.31	174	0.17	0.95	0.78(0.57-0.89)	0.99(0.96-1.0)	110.4	120.5

N = number of animals tested

Efficiency = (true positives + true negatives)/N

LR⁺ = positive test likelihood ratio = sensitivity/false positive rate

χ^2_1 = Chi square statistic with one degree of freedom, all are significant (p < 0.05) and indicate the statistical validity of each evaluation.

PR Test data interpreted using the cutoff derived from the negative Northland sera

LR Based on logistic regression model.

♂ = Males; ♀ = females; m = mature; j = juvenile

The B/C stimulation index of the LTA seems to provide the best sensitivity (78%) of any of the univariate assay methods, and although still not high was considerably better than any of the ELISAs. It did not appear that sex had any influence on the results of the LTAs. The logistic regression models using the LTA data showed slight improvement in sensitivity over the other methods of cutoff determination.

Agreement between the results of all assays, using the ROC cutoffs, is presented in Table 9-VIII. The level of agreement was poor to moderate for all of the test comparisons.

Table 9-VIII. Results of tests of agreement between assay results where sufficient samples existed for valid comparisons

Assay	N	Kappa	95% CI	t-value ^a
MPB70 vs CF	335	0.53	0.31-0.76	10.3
MPB70 vs Blocking	63	0.19	0.0-0.54	2.2
Blocking vs CF	44	0.12	0.0-0.46	0.8
B/C vs B/A	174	0.55	0.35-0.75	7.7
B/C vs MPB70	132	0.31	0.0-0.67	4.2
B/C vs CF	128	0.49	0.05-0.92	6.4
B/A vs CF	128	0.49	0.05-0.92	6.4
B/A vs MPB70	132	0.42	0.0-0.67	4.2

a values over 1.96 significant at the 95% level of confidence

Relationship to disease status

The number of gross and microscopic tuberculous lesion sites was known for 59 possums which had corresponding CF and MPB70 test data, and for 31 with corresponding Blocking ELISA data. Summary statistics for the number of lesions in these animals are presented in Table 9-IX.

Table 9-IX. Summary statistics of lesion numbers in tuberculous possums assayed by the three indirect ELISAs

Assay (n)	Lesion type	Mean (95% CI)	SD	Median	Range
CF and MPB70 (59)	Gross only	3.9(3.1-4.7)	3.1	3	0-15
	Gross plus ^a	9.5(7.6-11.4)	7.1	8	1-30
Blocking (31)	Gross only	3.9(2.9-4.8)	2.58	4	0-9
	Gross plus ^a	10.8(8.4-13.1)	6.43	11	1-23

a gross plus microscopic lesions

The difference in the median number of lesions between test positives and test negatives, using the ROC cutoffs, was tested for significance using the Mann-Whitney U test. No significant difference was found for the Blocking or the

MPB70 tests but the difference was highly significant for the CF test, those with more lesions having a greater chance of being test positive (Table 9-X).

Table 9-X. Results of the Mann-Whitney U test comparing the median number of lesions in test positive and test negative tuberculous animals assayed with the three indirect ELISAs

Assay	Lesion type	Test negative	Test positive	p-Value
CF	gross only	43	16	0.001
MPB70	gross only	50	9	0.27
Blocking	gross only	17	14	0.33
CF	gross plus ^a	43	16	0.001
MPB70	gross plus	50	9	0.19
Blocking	gross plus	17	14	0.91

a gross plus microscopic lesions

Histograms of the distribution of total lesion frequencies between test positive and negative individuals (Figure 9-4) reveals that this difference in medians is due to a large proportion of the test positive animals (particularly in the CF, but also MPB70 assays) having the bulk of the lesions, whereas in the Blocking assay the number of lesions is more evenly distributed. The Blocking assay results appear to be independent of the number of lesion sites affected.

Examination of the records of infected animals which were subjected to the LTA allowed the status of these possums to be divided into three categories i.e. 1) those which were known to have substantial lesions, and 2) those which had no gross lesions or had superficial lesions which had recently drained, and 3) those for which the exact lesion status was indeterminate. Contingency table analysis was performed to compare the test results, for the two categories for which lesion status data was available ($n = 24$). A significant difference (Fisher's 2-tailed $p = 0.002$; OR; $2.5 < 72.0 < 3722$) was found between the two groups suggesting that the LTA failed to provide evidence of disease in animals in which gross lesions were absent or in those which had drained and released antigenic material.

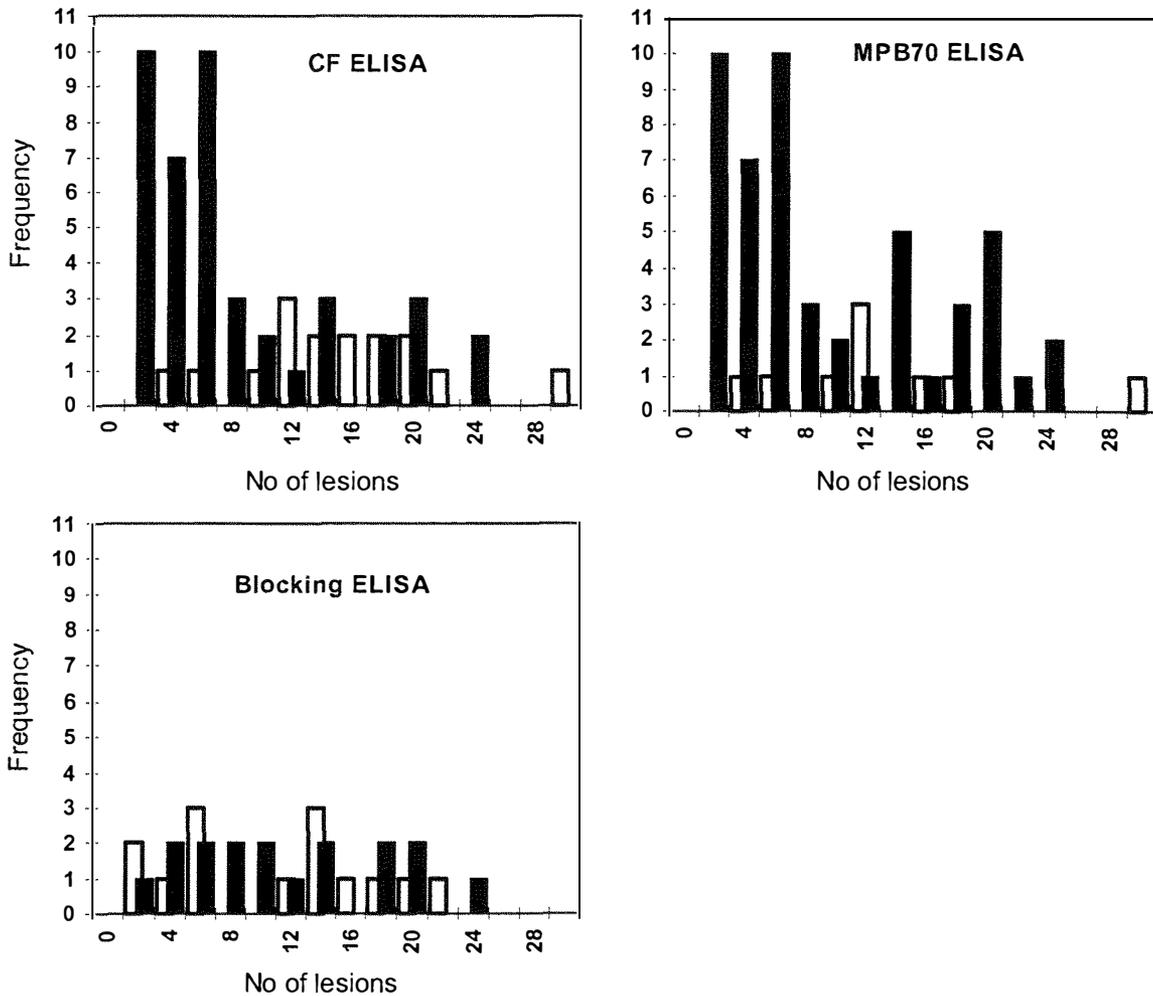


Figure 9-4. Histograms showing frequencies of test positive (□) and test negative (■) sera from diseased possums, categorised by total number of lesions, gross plus microscopic

Results of the paired t-test on the repeated measures of CF and MPB70 ELISAs performed on sera from 14 possums injected with tuberculin, showed that there was a significant (two-tailed $p \leq 0.001$) rise in circulating antibody level at 4 to 5 weeks post-injection, in both assays. With the CF ELISA, 13 of the possums had increased antibody levels (range -0.8 to 23.2 OD units), with four of these being over 10 OD units. This rise was not sufficient however to lift any CF titre above the cutoff level for a positive test diagnosis, despite one of the possums being infected, and having multiple lesions. The MPB70 antibody levels of all 14 possums rose (range 0.1 to 24.1 OD units) in response to tuberculin priming. Three possums, not including the infected individual, showed rises greater than 10 OD units, although again none rose above the cutoff level for a positive diagnosis.

Longitudinal study possums

There were six methods used to make positive diagnoses i.e. culture, histology, necropsy, clinical exam without ancillary tests, LTA, and ELISAs. The number of possums examined was 979, which included those from the termination which had not previously been examined. Isolation of *M. bovis* from lesions or pooled nodes was the definitive diagnostic method, and was employed in as many possums as possible. Gross necropsy and histology were viewed as being the next most reliable means of disease diagnosis, followed by clinical evidence, LTA, and ELISA results, in that order. On applying these hierarchical criteria for disease diagnosis, isolation of *M. bovis* was the criterion used to establish a diagnosis in 370 possums and found disease in 100 cases. Necropsy alone was employed in 192 possums and found disease in three cases. Histological examination revealed two of 18 animals infected. Clinical examination diagnosed tuberculosis in five of 102 possums. LTA identified disease in 56 of 121 possums and ELISAs provided evidence of infection in 22 of 176 animals. Overall the use of ELISAs or LTA allowed the identification of an additional 79 apparently infected possums, in addition to the 109 which were already known to have been infected.

Blood test results from possums examined during the longitudinal study were examined using the best cutoff (Table 9-VII) derived from the ROC curves for the appropriate univariate (Blocking ELISA) and multivariate (B/C, B/C-B/A, MPB70^{LR}, and CF^{LR}) analyses. The results of this and other related observations on these possums are presented below.

The Blocking ELISA, using the cutoff value of 0.22, derived from the initial ROC analysis was applied to 1477 sera from the longitudinal study. The sera from 362 individual possums were tested, with up to 15 tests per individual. Tuberculosis was diagnosed in 29 animals tested. There were eight animals which had multiple positive tests, with the most being 3 out of 10 and 3 out of 6 tests positive. Infection with tuberculosis was confirmed by culture in nine of the 29 possums. Nine of the possums were lost to follow up, five disappeared within 3 months of the positive test, and the remainder were lost at 6, 8, 9 and 36 months afterwards. The remaining 11 were found to be tuberculosis-free as a result of necropsy and pooled lymph node culture, which was carried out over 3 years after the positive test in nine cases, and at 2 and 8 months post-test in the remainder.

The MPB70 ELISA, using the logistic regression model, and the cutoff derived from ROC analysis (0.86), was applied to 1281 sera from the longitudinal study. The sera were collected from 540 possums, and the number of samples per animal varied from 1 to 15. Tuberculosis was diagnosed in 14 of the animals tested, with 2 of the possums having two test positive episodes. Infection with tuberculosis was confirmed by culture in six of these possums (including the two with dual positive tests). Three were lost to follow up, two at 1 month after the positive test, and the other one 2 years later. The remaining five were found to be tuberculosis-free as a result of necropsy and pooled node culture, two at the time of the positive ELISA test, and the remainder 3 to 5 years following the positive test.

The CF ELISA, using the logistic regression model and the cutoff derived from ROC analysis (0.64), was used on 310 sera from 259 possums. Three possums were test positive, with two testing positive once and only one returning two positive tests. Each of these three animals were infected with *M. bovis*.

The LTA was applied to 745 blood samples from 323 possums with up to 8 tests per possum being performed. Over 100 test results were discarded because assay reliability criteria had not been met. Eighty one possums were diagnosed as tuberculous using results of the LTA predictive models. There were 19 possums with multiple positive test episodes, with five out of six being the most positive results any individual achieved. Tuberculosis was confirmed in 25 of the LTA positive possums, with 11 dying from tuberculosis within periods ranging from 2 to 42 months (median = 13.7 months) post-diagnosis. Seventeen test positive animals were lost to follow up at periods ranging from 1 to 36 months (median 8.5 months) after the first positive test. The remaining 39 were found to be free of tuberculosis following necropsy and culture of pooled lymph nodes, at periods ranging from 0 to 54 months (median = 29.8 months) post-test. Comparison of the time to death or disappearance, of the known tuberculous possums and the group lost to follow up, using survival analysis showed that the mean and median survival time from date of birth, of the 17 possums lost to follow up was 10 (6-15) and 8 (4-12) months respectively, whereas the mean and median survival period for the 25 which were confirmed as tuberculous was 27 (17-37) and 22 (13-31) months respectively. The time of survival for the possums lost to follow up was significantly shorter than for

those possums in which tuberculosis had been confirmed (Logrank = 8.75; $p = 0.003$).

Of 189 possums diagnosed as tuberculous, 47 of these were diagnosed at death (24.9%). Ten of these animals had been examined within the previous 2 months, and thus had an opportunity for ante-mortem diagnosis based on clinical examination. Of the remaining 142 possums with tuberculosis diagnosed, 63 were diagnosed infected through clinical examination and/or culture of aspirates, and 79 were diagnosed by blood tests. Although 152 possums had an opportunity for clinical diagnosis (many on multiple occasions) only 63 were found to be tuberculous by this method, thus showing an apparent sensitivity of this method of 0.41 (0.34-0.49).

After the 1st June 1993 when the necropsy and specimen collection procedure was known to be consistent, there were 355 possums necropsied, from which appropriate samples were submitted either for mycobacteriology and/or histology. Of these there were 311 samples from possums with no gross lesions, and of these nine were culture or histology positive. There were 44 possums diagnosed as tuberculous at necropsy, all of which were culture or histology positive. The apparent sensitivity of gross examination was thus 0.83 (0.70-0.92), and the specificity 1.0 (0.98-1.0), by comparison with the examination by culture or histology. There was one isolation of *M. avium* from an animal with no gross lesions.

Of the 100 animals diagnosed tuberculous by bacteriology, 22 were also found to have positive LTA, and 19 also had positive ELISAs at some time. Of the 270 found to be negative by bacteriology, there were 145 which were also negative by LTA. One animal each diagnosed by necropsy, histology and clinical exam also had a positive LTA. Of the 56 possums with positive LTA there were 3 which also had positive ELISAs. Note that these tests were not necessarily performed at the same time on each of the animals. There were 97 possums for which no diagnostic test results apart from clinical examination were available.

At the termination, where 189 previously trapped possums were necropsied, and from which pooled lymph nodes were submitted for culture, there were 20 possums which had at least one positive LTA at some time, and 7 possums for which there

had been at least one positive ELISA test, but which at necropsy showed no evidence of tuberculosis.

Kaplan-Meier survival curves for each stratum of the total population are presented in Figure 9-5. Of the 979 possums examined, observations were right censored for 460 possums killed either through or at the termination of the study, and also for 92 animals which suffered accidental deaths, principally related to cardiac puncture. For confirmed tuberculous possums, there were 72 failures and 38 right censored observations. The mean and median survival periods for tuberculous possums were 46 (40-51) and 41 (36-46) months respectively. For those which were positive to a blood test there were 35 failures and 43 right censored observations. The mean and median survival periods were 75 (64-87) and 83 (66-100) months respectively. In the third category of possums, which were believed to be disease free, and which were known to have died, there were 65 failures and 471 censored observations. The mean and median survival periods were 90 (80-99) and 102 (91-113) months respectively. In the last category, of apparently disease free individuals which were all lost to follow up, there were 255 failures, with a mean and median survival time of 26 (24-29) and 19 (16-22) months respectively. The stratified logrank test over all pairs pooled showed that the four curves were significantly different from each other. The χ^2 statistic varying between 422.2 ($p < 0.001$) for the comparison of the two non-tuberculous groups, and 8.3 ($p = 0.004$) for the comparison of the test positive group and the non-tuberculous group which were known to have died.

Long term survival of a few infected possums was supported by evidence from five LTA positive possums which survived for 19, 22, 26, 28 and 42 months after having the first positive test, before dying from confirmed tuberculosis. Four of these possums with multiple LTA results showed negative test results after the initial positive tests, suggesting the possibility of a period of disease quiescence during the infection, followed by recrudescence and death. The restriction endonuclease (REA) types identified from these possums provided no evidence that the isolates had been acquired recently before death.

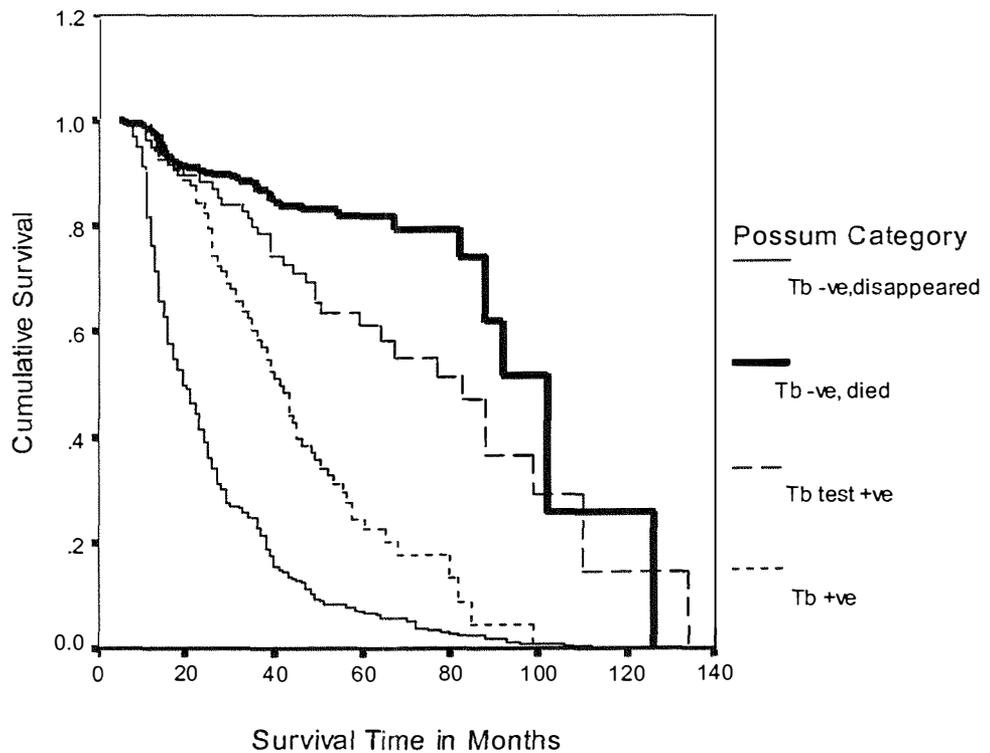


Figure 9-5. Kaplan-Meier survivor function for infected (stratified by diagnosis) and non-infected possums (stratified by cause of failure), based upon the age of the possum

In 16 instances mature females which were known to be infected, or suspected of being so, through having either a positive ELISA or LTA test result, also had known offspring which were similarly known or suspected of being infected. In 11 dams the time of their apparent infection appeared to coincide with the period in which the offspring of interest was closely associated with the mother. In four cases where diagnosis was by isolation of *M. bovis* from both the mother and offspring, the infecting REA type involved was the same. Three of these offspring, which died naturally from tuberculosis, were 9, 12 and 13 months of age at death. In two other dams which were diagnosed by isolation of *M. bovis*, one which died from the disease had an offspring which had a positive Blocking ELISA 2 months prior to disappearing at 11 months of age, and the other mother which died accidentally, had an offspring which was LTA positive at 9 months, but apparently free of tuberculosis when killed at 42 months of age. Four dams which were LTA positive produced offspring which were also LTA positive in at least one test. These offspring were first positive between 11 and 13 months of age, but apparently free of tuberculosis when killed between 25 and 43 months old. Mothers from which *M. bovis* was isolated, or had a positive ELISAs ($n = 7$) were significantly more likely

to have offspring which would die from tuberculosis or disappear (presumably tuberculosis deaths), than mothers ($n = 4$) which were diagnosed positive by LTA only (Fisher's exact 2-tailed test $p = 0.015$).

During the years 1991/92, 1992/93, 1993/94, and 1994/95, commencing at the beginning of April and finishing at the end of March each year, the number of positive LTAs was, 72/204 (35.3%), 18/195 (9.2%), 5/130 (3.8%) and 12/216 (5.6%) respectively. During 1991/92, when 35.3% of all LTAs were positive, conventional techniques, involving clinical examination and necropsy, over the same period, identified 22 tuberculous animals from 245 possums examined (9.0%).

Among the LTA positive possums, the high and low B/A SI groups had a mean survival time of 61 (53-70) and 66 (51-81) months respectively, and a median survival time of 59 (44-74) and 53 (39-67) months respectively. The logrank χ^2_1 of 0.06 was not statistically significant ($p = 0.81$).

The relationship of infection prevalence between possums with and without gross lesions (NGL) is presented in Table 9-XI. Diagnosis of infection in NGLs was made by culture of pooled nodes, or extensive histopathological examination. A significant trend ($\chi^2_2 = 23.9$, $p < 0.001$) was found for there to be a greater prevalence of infection in NGL possums within populations in which a higher prevalence of gross disease was found. The ratio of NGL possum prevalence to grossly diseased possum prevalence was close to 1:2 for each of the three prevalence categories.

Table 9-XI. Summary data on investigation of relationships between the prevalence of grossly identifiable disease and prevalence of infection in possums with no gross lesions (NGL)

Prevalence category	N	Gross prevalence	NGL examined	NGL positive	NGL prevalence	Ratio NGL/gross ^a	Odds ratio ^b
0 to 10%	721	0.040	426	9	0.021	0.525	1.00
11 to 20%	187	0.155	56	4	0.071	0.458	3.38
>20%	68	0.515	19	6	0.316	0.613	14.95

a ratio of prevalence of infected possums without gross lesions to prevalence of those with gross lesions.

b Odds ratio derived from the χ^2 analysis for trend, investigating the relationship between prevalence of NGL infected possums and the prevalence of gross lesions.

Case histories

Individual case histories from selected possums which show characteristics which are different from “normal” infected or disease-free animals, are presented below, to draw attention to particular aspects of the epidemiology of tuberculosis which these individuals help clarify.

Infected possums without gross lesions.

Case 3747 was a mature male estimated to be over 2 years of age when he arrived on the study site in October 1989. This animal first provided evidence of infection when tested by LTA in February 1992. The possum was negative to 9 blocking ELISAs, and 8 MPB70 ELISAs on serum collected between October 1989 and November 1991, and was also negative to a LTA conducted in April 1994. The possum was regularly caught, and was found dead in a cage in August 1994. At necropsy both deep axillary lymph nodes were slightly enlarged and had a granular appearance. Although tuberculosis infection was not suspected, *M. bovis* was isolated from the pooled nodes collected at necropsy. REA typing showed this to be strain which has not been identified in any other animal on or adjacent to the study site, despite widespread examination of multiple species within about 12km of the study site, in order to characterise the REA types in the area (chapter 12). It appears that this possum was sub-clinically infected when it dispersed to the study site from some distance away, and maintained the infection during 5 years residence on site. The only other recorded isolation of this REA type has been from two animals, a possum and a ferret, found 2 and 3 km, respectively, to the west.

Case 2393 was a female, approximately 15 months old when first captured in September 1989. The first evidence of infection was found at clinical examination in December 1991, when a suppurating sinus was detected over the area of the mandibular lymph node. This draining lesion persisted on and off till November 1993. Despite several attempts at mycobacterial isolation no *M. bovis* were recovered, but a pseudomonad was isolated on one occasion following aerobic culture. The first LTA in July 1992 was positive, but three subsequent tests, including the one at slaughter in September 1994 were each negative. Twelve blocking and 11 MPB70 ELISAs performed on sera collected between September 1989 and December 1991 were all negative, as was a CF ELISA carried out on sera collected at slaughter. Although the carcass showed no gross lesions at necropsy,

M. bovis was isolated from pooled lymph nodes. In this case it seems plausible that the initial draining lesion was probably tuberculous, but that as immunity developed to *M. bovis*, a secondary bacterial invader took its place and caused the lesion to persist for a protracted period. Alternatively, the mandibular node lesion was unrelated to the *M. bovis* infection which remained subclinical.

Case 2304 was a female, approximately 13 months old when initially captured in June 1993. She was trapped on the southern side of the study site, where there were no known tuberculous possums resident, and clinical examination found nothing remarkable at time of first capture. She was not re-trapped until the termination, when she was caught just off-site, once again on the southern side. Necropsy in October 1994 found no gross lesions, however culture of pooled nodes showed that she was infected with *M. bovis*. CF and MPB70 ELISAs performed on sera collected in October provided no evidence of infection. It is thought likely that this possum acquired infection elsewhere, possibly on the northern side of the study site before first capture, as there appeared to be little risk of acquiring infection in the area in which she was resident. She may well have been progeny of an infected dam from the northern fringe of the study site, where infection was endemic with her REA type.

Three other cases of infection in possums without gross lesions were found in possums at or around the time of the termination. The possums were estimated to be 2.5 years of age or older, and each had been captured at least once prior to necropsy but clinical examination at that time revealed no evidence of infection. Each of the possums were trapped prior to necropsy, in an area in which there were known to be tuberculous possums present, and each had *M. bovis* isolated from pooled lymph nodes, but we were unable to determine the likely time of infection.

At the termination there were also three other possums found to have no gross lesions, but from which *M. bovis* was isolated in two cases and AFB found in another. None of these animals had been captured previously. One of these with an *M. bovis* isolate was a 4 year old female which was caught in an area with prevalent tuberculosis. The other possum from which *M. bovis* was isolated was a 3 year old male which was captured on the southern side, away from likely nearby contact with other tuberculous possums. Both of these possums were negative to the CF and MPB70 ELISAs at the time of necropsy. The possum in which AFB were

detected during extensive histopathological examination, was a 2 year old female captured again in the area with prevalent tuberculosis. Lesions in this animal were found in the right superficial axillary lymph node, and consisted of two small areas of macrophage accumulation, in one of which a single AFB was visible.

Details on a further two possums which were without gross lesions at necropsy, but which had histopathological evidence of infection were available. These animals came from the Flagstaff area in 1992. Both of these were mature females subjected to extensive histopathological examination, and each showed evidence of disease in one inguinal lymph node only. Both had small pyogranulomatous lesions, without necrosis, containing AFB in moderate and large numbers respectively.

Possoms showing evidence of lesion resolution or regression

Case 2191 was a male possum, first captured in October 1992 at approximately 1 year of age. The first evidence of disease was from clinical examination in January 1993, during which an enlarged deep axillary lymph node was palpated and subsequently found to contain caseous material, from which *M. bovis* was isolated. This large lesion remained evident up until slaughter in September 1994. LTA, CF and MPB70 ELISAs conducted in June and September 1994 were all positive for tuberculosis. At necropsy a 5 by 4 cm firm caseous lesion encapsulated by a thin fibrous membrane was the only gross lesion detected. Histologically this lesion was found to contain very few AFB. There were also tiny residual granulomas in both the lung and bronchial lymph node, both without AFB. Mycobacteriological confirmation of continued infection was not conducted following necropsy. The lung lesions may have been more severe earlier in the infection, but resolved, possibly as a protective immune response to tuberculosis developed. The axillary lesion was clearly too large to be resorbed, and so remained encapsulated.

Case 2285 was a male, first captured in March 1992 at an age of 6 months after being born to a known LTA positive mother which disappeared in May 1993. First evidence of tuberculosis was through detection of a slightly enlarged axillary lymph node in December 1993. Tuberculosis was later confirmed in June 1994 after aspiration of caseous material from a grossly enlarged inguinal lymph node. Three LTA conducted between November 1992 and May 1993 were negative, but a fourth test taken at necropsy in September 1994, was positive, as were CF and MPB70

ELISAs at this time. At necropsy there were multiple lesions visible in five lung lobes, but with evidence of pleural cicatrization and tubercle contraction. Lesions were of a pyogranulomatous nature, containing small nodules of caseous material. AFB were rare in the lungs and in other lesions involving the axillary and inguinal lymph nodes. The inguinal nodes were the only other site to contain caseous material. The overall impression gained from this animal was one of long standing and severe tuberculosis, but in which the disease process had been arrested and resolution of lesions begun.

Another similar case to 2285 was No. 5724, first captured in December 1994. This was a male approximately 4 years of age. At second capture in February 1995 a draining lesion of the left superficial axillary node was detected. This lesion was small, covered with a scab and contained a tiny quantity of caseous material from which *M. bovis* was isolated. Lesion development and drainage had occurred over the previous 9 week period. At later examinations there was no sign that there had been any disease of the axillary lymph node and the possum remained in good health. The animal was killed by the neighbour in October 1995, and was necropsied after thawing of the carcass. The only gross lesion apparent at necropsy was the consolidation of one diaphragmatic lung lobe, which contained several caseous foci 3 to 4 mm in diameter. Histopathology of the lung revealed that the lesion was principally composed of epithelioid cells with some fibroblasts. Necrosis was rare, as were AFB. The gross appearance of the lung was similar to the one depicted in Figure 9-6, which came from a possum shot off-site. The overall impression of this case was also of an animal with a severe disease of long duration, but in which the cell-mediated immune system had prevailed and lesion resolution or regression was well advanced.

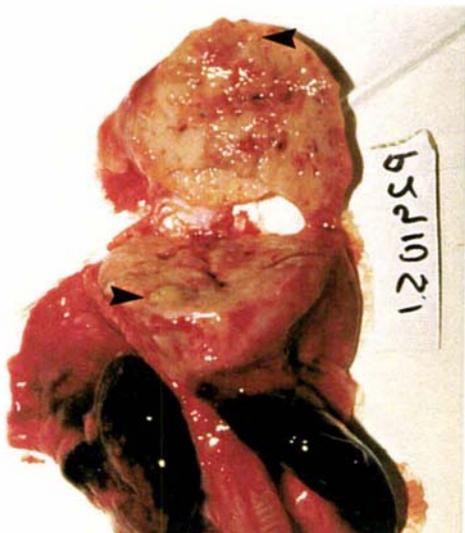


Figure 9-6. Lung from possum showing evidence of resistance. Sectioned consolidated lung lobe, showing parenchyma replaced by a solid mass of granulomatous tissue in which pockets of necrosis are visible (arrowheads).



Figure 9-7. Tuberculous superficial axillary lymph node from the same possum, showing a 'pea' of consolidated caseous exudate surrounded by a thick capsule of pyogranulomatous inflammatory tissue.



Figure 9-8. Sectioned inguinal lymph node showing the more typical appearance of a grossly affected node. Note the very thin capsule remaining, which surrounds the large mass of glutinous necrotic tissue.

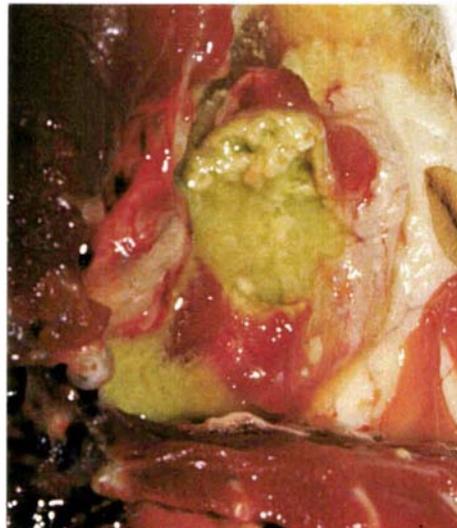


Figure 9-9. Enlarged tuberculous deep axillary lymph node, sectioned to show the liquid necrotic content which also commonly replaces the lymphatic tissue.

Case 38484 was a male of approximately 15 months age, captured and necropsied at the termination. This possum possessed an unusual single gross lesion of the left superficial axillary lymph node. The node was enlarged and contained a free pea-like mass of caseous material which shelled out readily from its encapsulating mantle, and was very similar in appearance to the axillary lymph node depicted in Figure 9-7. Histopathology showed that the capsule was composed of pyogranulomatous tissue without visible AFB. There were also focal macrophage accumulations in the deep axillary and hepatic nodes with only a few AFB found in the deep axillary node. This possum was LTA positive, but negative to the CF and MPB70 ELISAs.

Five other possums had single gross lesions. Two of these were males aged approximately 15 and 24 months, and each possessed an inguinal or superficial axillary lesion which had drained through the skin, and all that remained was tiny focus of purulent exudate covered by a scab. In both of these cases there were other microscopic lesions which primarily contained only macrophages. AFB were rare in all lesions. The animal with the inguinal lesion was LTA negative, but the other individual was LTA positive. The third possum had only one lesion in the inguinal lymph node which contained a 2 by 2 cm mass of caseous material, which if it had burst, drained and healed, would have left the possum without any histological lesions whatsoever. The other two cases, both mature males, showed single 5 to 10 mm lesions in the diaphragmatic lung lobes. Microscopic lesions were also found in axillary and inguinal lymph nodes, the spleen in one and the tonsil of another, thus suggesting that the lung may have been a primary lesion, and the other sites, (barring the tonsil) being infected haematogenously. One of these two possums had blood subjected to LTA with a positive result. All the above five possums were CF and MPB70 ELISA negative.

Case 3510 was a female of 10 months age when first captured in June 1989, which lived in a part of the study site in which tuberculosis was prevalent. The first evidence of disease was in October 1989, when a slightly enlarged right axillary lymph node was palpated. In May 1990 a some enlargement of a left axillary node was also detected. One of the right axillary nodes was later found enlarged with a 7mm diameter in April 1992. The left deep axillary was again slightly enlarged in April 1993. No attempt was made to aspirate material from these enlarged nodes as

the slight increase in diameter was considered non-specific on each occasion. This animal however did have multiple LTA positive tests. The first LTA in December 1991 was negative, but a subsequent test in February 1992 was positive, as were three others, including the last test in June 1994. Twelve blocking ELISAs and 13 MPB70 tests carried out on sera collected between June 1989 and June 1994 were all negative, as was a CF ELISA carried out in June 1994. One offspring, born in April 1991 also showed LTA evidence of infection when 11 months old, but was apparently free of infection when killed at 43 months of age. Possum 3510 was trapped, killed and skinned by the neighbour in August 1994. Necropsy of the thawed carcass, and pooled node culture failed to provide evidence of infection. It appears with this case that the animal may possibly have become infected in late 1991. If so, whatever lesions drove the positive LTA and produced mild superficial lymphadenitis must have resolved completely. If the animal was truly infected the failure to isolate *M. bovis* may have been due to low numbers of bacilli being present at death, or perhaps those present may have been dormant and unable to grow, or alternatively the organisms may have been present only in the superficial axillary, or inguinal nodes which were removed with the skin and consequently not examined or submitted for culture. The true status of this animal remains uncertain.

Case 2462 was a male trapped for the first time in May 1989, when approximately 19 months old. The first evidence of tuberculosis was from a positive blocking ELISA test conducted on serum taken in February 1990. Eleven other blocking ELISAs and 12 MPB70 ELISAs carried out on sera collected over the period December 1989 to November 1991, were all negative, as were a CF and an MPB70 ELISA conducted in June 1994. The first LTA in May 1991 was negative, but five subsequent tests concluding in June 1994 were all positive. The animal was found dead in August 1994, slightly decomposed but suitable for necropsy. There were no lesions suggestive of tuberculosis, and the animal appeared to have died from exposure/starvation. Pooled lymph node culture provided no evidence of infection. As in the previous case it is possible that disease had been present, but that lesions had resolved and there were too few viable bacilli present at necropsy to allow successful culture.

Discussion

The use of ROC curves for evaluation of medical tests has been discussed and advocated by numerous authors including Erdreich and Lee (1981), Swets (1988), Fletcher, *et al.* (1988), Begg (1991), Kraemer (1992) and Hasselblad and Hedges (1995). The main advantages of the method are, that, unlike conventional techniques for determining sensitivity and specificity for a test, it is independent of prevalence and furthermore is a non-parametric procedure which makes no assumptions about the statistical distribution from which the data was drawn. Each point on the curve corresponds to a numerical test result, which if taken as the cutoff point between normal and abnormal, yields the sensitivity and specificity values corresponding to the point's co-ordinates. The perfect test has an area under the curve of 1.0, while a non-informative test has an area of 0.5. The experience from this study was that the ROC analysis was computationally easy to perform and had less attendant problems than the alternative method.

The performance of the 3 ELISAs was disappointingly poor and was exacerbated by inconsistency between test results from serially collected samples. When the Blocking and MPB70 assays were performed on the longitudinal sera, only three sera from the true positives were positive to both assays. Serially collected sera from known positive animals did not always test positive and the immunological processes operating in these animals clearly did not maintain high antibody levels over time. Both host genetic variability modifying the humoral immune response to specific *M. bovis* antigens (Griffin *et al.*, 1991), and differences in the capacity of *M. bovis* strains to secrete MPB70 (Harboe and Nagai, 1984), may be partly responsible for the poor observed performance of the ELISAs. Overall, the study clearly demonstrated that when high specificity is required the assays could not reliably detect infection and they therefore have limited epidemiological or practical value. There may be some prospect however, of furthering the limited evaluation of the Blocking assay. This test shows the most promise, as it appears to have the highest sensitivity (with specificity set high) and is the one most likely to show a positive serological response in animals with fewer lesions, which by implication are at an earlier stage in the disease process. In previous studies in cattle and deer positive serological responses to *M. bovis* infection appeared to be found more frequently in advanced or disseminated disease (Lepper and Corner, 1983; Ritacco *et al.*, 1991; Griffin *et al.*, 1991) and the results of this study and of Buddle *et al.*

(1995), and Jackson (1995) suggest that the same is true for possums. The low to moderate agreement between comparisons of pairs of univariate tests indicated that there was a possibility of increasing the sensitivity of testing by parallel interpretation of combined tests. This may be true for combinations of the ELISAs (Lugton *et al.*, 1995), but was not found to be the case with combinations of ELISAs and LTA when examined by logistic regression modelling. Parallel interpretation of the Blocking assay with the CF ELISA could conceivably furnish a test with moderate utility.

All three ELISAs had low sensitivity when cutoff points were selected to maximise specificity. Tests with low sensitivity and high specificity may cause serious under-estimation of true positives. As prevalence decreases, the ratio of false positives to true positives increases; an effect which is most marked at a combination of low levels of sensitivity and low disease prevalence, as was the case when many of these tests were applied to the longitudinal study populations. Thus it would be expected that a number of the ELISA positive responses from animals which were lost to follow up, or culture negative at necropsy were false positive diagnoses. This is illustrated by the MPB ELISA positives, two of which were culture negative on the day the test positive serum samples were collected.

Test evaluation ideally uses naturalistic sampling which involves a diagnosis and test for all animals in the population of interest. Although conceptually attractive, it is difficult to perform in practice. The method was able to be followed here by using detailed diagnostic procedures involving necropsy and laboratory testing of all cross-sectionally sampled animals. The problem with the method is that it ideally requires a balanced ratio of test positive and test negative samples, and a large total sample size. The degree of imprecision encountered was evident in the width of the confidence intervals for the estimates of sensitivity and specificity calculated using this method.

Pseudo-retrospective sampling involves sampling both from a known diseased population and from a population known to have very low or zero prevalence (such as the Northland possums). It is an attractive method mainly because it is logistically simple and relatively cheap to implement. The problems which attend this method are that two separate populations are involved, a high risk and a low risk group, neither of which may be representative of the population of interest in

which the test will be applied. Another difficulty is that both specificity, and to a lesser extent sensitivity, are both known to vary between populations. Despite these caveats which apply to these sampling techniques, the results produced in this study were comparable for the CF and Blocking ELISAs, but use of the cutoff derived from the Northland sera, in the case of the MPB70 ELISA, doubled the sensitivity compared with that derived from the ROC, but also reduced specificity slightly. This may indicate that the Northland tuberculosis-free population is not comparable to the populations from the Wairarapa and West Coast, possibly because there is a higher level of antibodies to this *M. bovis* specific antigen, in possums from an infected population.

Serological tests using crude antigens, such as the CF ELISA, not only lack sensitivity (because of low antibody levels in infected individuals), but also suffer from low specificity due to the cross-reactive nature of the antigens. Indeed this cross-reactivity may have been responsible for the rises in serum antibody following exposure to injected tuberculin in the 14 possums. Increase in antibody levels following tuberculin priming has been demonstrated in cattle (Ritacco *et al.*, 1987; Hanna *et al.*, 1992; Costello *et al.*, 1997) and deer (Griffin *et al.*, 1994). As the MPB70 antibody antibodies also rose subsequent to the tuberculin priming, and substantially so in 3 cases, suggests that some apparently non-infected possums may have had an anamnestic response to previous exposure to *M. bovis* antigens or that that the MPB70 used was cross-reactive with other bacterial antigens (Wood *et al.*, 1992; J. F. T. Griffin pers. comm.).

Improvement in the detection of serological responses to *M. bovis* in possums may have to await the development of ELISAs using a cocktail of more discriminating, *M. bovis* species specific antigens e.g. MPB70, A60 antigen complex, ESAT 6 (Fifis *et al.*, 1994; Saegerman *et al.*, 1995; Buddle, pers. comm) or the 26 kDa antigen reported by O'Loan *et al.*, (1994), which would serve to improve the specificity and sensitivity of the tests. Another option for improving specificity, and which is currently feasible, is the incorporation of both avian and bovine ELISAs in the test protocol, as has been done successfully in the case of ELISA testing for tuberculosis in deer (Griffin *et al.*, 1994). Test positive animals are those which have antibody levels to *M. bovis* antigens substantially higher than those to *M. avium* antigens.

The predictive models showed that sex, age and lactational status have little influence on the test outcome. The apparent effects of age and sex on the initial univariate ROC-derived cutoffs suggested that sera from males and juveniles in the CF and Blocking ELISAs are more likely to produce a reliable result. However, this conclusion is probably spurious, judging by the outcome of the logistic regression models and the small differences in the area under the ROC curves (which have wide confidence intervals). An interesting, but inexplicable, finding was that the condition index of possums tested with the MPB70 assay had a significant influence on the outcome of the test, whereby infected animals in better condition were more likely to show a positive result.

Although handicapped by low numbers of possums in the evaluation, the LTA showed the most promise, being a usefully sensitive test (approx. 80%), when the specificity was set high. The regression models of B/C SI alone or interpreted in combination with the B/A SI were clearly the best available tests to establish the true infection status of possums. The limited data available suggested that significant antigenic stimulation, from gross lesion presence was necessary to drive the lymphocyte mitogenic response, as LTA from infected possums were less likely to be positive in animals in which no gross lesions, or drained lesions were found.

Survival analysis needs careful interpretation as the non-tuberculous groups have been split on the basis of whether they were lost to follow up or not. The analysis conducted was found to be the most satisfactory, as a large proportion of the non-tuberculous possums which disappeared, especially in the first 20 months of life were thought to have been lost through emigration. Analysis of the two non-tuberculous categories combined, produced a curve that declined more sharply than that of the possums which were known to have tuberculosis, which is clearly likely to be a spurious result. Because of this, and the belief that the vast majority of failures in the tuberculous possums will have been due to death from tuberculosis, the data is presented "as is". The survival curves show that confirmed infection is responsible for a reduction in the life expectancy of possums, whereas the suspected presence of infection, evident by LTA or ELISA is associated with an increased risk of death but with a median survival time twice that seen with clinical disease. This suggests that the tests are inaccurate in a large number of cases, or alternatively, and more likely, that sub-clinical disease is less directly linked temporally to the death of infected possums. Furthermore the data arising from the survival analysis

suggests that the disease normally runs a prolonged course, with a median survival age in confirmed and LTA positive cases of 41 and 83 months respectively. The one possum which is known to have died from tuberculosis in the Orongorongo Valley, possibly brought the infection from several kilometres away as a migrating juvenile (Brockie *et al.*, 1987). This possum had been resident on site for at least 3.5 years, and died at an estimated age of 5 years 4 months, thus suggesting a long pre-clinical disease period, consistent with our findings. Approximately half of the LTA positive possums were apparently *M. bovis*-free at necropsy, suggesting that a substantial number of exposed possums fail to have the infection establish, or eventually resolve any lesions which may have developed.

The occurrence of pseudo-vertical transmission was also supported by the findings of the LTAs and mycobacteriology. Although the data is limited, it appears that offspring infected by dams with clinical disease are likely to die around 12 months of age, whereas those apparently infected by dams with subclinical (LTA positive) disease are less likely to succumb to the infection.

As it had been suggested that animals which mount a strong lymphocyte stimulation response to avian tuberculin may be reflecting an enhanced immunity to mycobacterial antigens in general (Griffin *et al.*, 1993a) survival analysis investigating differences between high and low avian PPD responders was performed on LTA positive possums. It was found, however, that there was no significant difference in the age at death between the high and low avian PPD responders, suggesting that cross-reactivity of peripheral lymphocytes to other common mycobacterial antigens did not confer any survival advantage to apparently infected possums.

LTA have been used previously to follow the course of disease in experimentally infected possums (Buddle *et al.*, 1994). Fourteen of the 15 intratracheally inoculated animals, which developed severe disease, showed strong LTA responses to bovine PPD. This was initially detected at 19 days post-inoculation in possums administered high and medium doses of *M. bovis*. Possums given 20 cfu (low dose) of *M. bovis* first showed strong responses some time between 19 and 33 days post-inoculation. Two in-contact controls showed high SIs to bovine PPD at 47 and 61 days post-inoculation, thus suggesting that they had become infected, although apparently lesion-free at necropsy and without *M. bovis* recoverable from their lungs. This suggested that the number of organisms in the lungs was low or that the

infection was sited elsewhere, and that it is possible to have a good peripheral lymphocyte response, with little antigen present, at least in the early period post-infection. Similar LTA responses, without apparent disease have been recorded in deer (Griffin *et al.*, 1991), and have been attributed to the development of resistance following infection with *M. bovis*.

Gross lesions in the inoculated possums which died or were killed at 60 to 64 days post-inoculation, were characterised by the presence of high numbers of AFB, and central necrosis with limited granulomatous reaction.

In possums successfully vaccinated with BCG (Aldwell, *et al.*, 1995a; Aldwell *et al.*, 1995b), pulmonary lesions in *M. bovis* challenged animals were characterised by a granulomatous response with little necrosis and few AFB and much larger numbers of macrophages than neutrophils, and the presence of some giant cells. This contrasts to the lesions in unvaccinated experimentally infected possums which show extensive necrosis, equal numbers of macrophages and neutrophils, rare giant cells, absence of epithelioid cells, high numbers of AFB (O'Hara *et al.*, 1976; Comer and Presidente, 1980; Comer and Presidente, 1981; Buddle *et al.*, 1994; Pfeffer *et al.*, 1994). The resistance to infection in these studies is likely to have been compromised by the stress of captivity (Buddle *et al.*, 1992). Typical necrotic lesions, characterised by liquefactive or caseating necrosis, seen in peripheral lymph nodes of most natural cases are shown in Figures 9-8 and 9-9. The appearance of these lesions is easily recognised as different from the encapsulated axillary lymph node lesion shown in Figure 9-7, which came from a possum which was apparently successfully resisting the disease.

In experimental studies, strong peripheral blood lymphocyte blastogenic responses to bovine PPD stimulation, has not shown a strong correlation with protection from disease (Aldwell *et al.*, 1995a; Aldwell *et al.*, 1995b). Eight of 13 intranasally vaccinated possums which showed no LTA responses following inoculation, still demonstrated a significant level of protection, which was similar to others in the same group, and to others which were vaccinated subcutaneously (all of which showed positive LTA responses). Eighteen of twenty *M. bovis* challenged (both intranasal and subcutaneous) possums showed LTA responses to bovine PPD, with SI greater than 3.0. Although Aldwell *et al.* (1995a) noted similar post-vaccinal SI responses in orally vaccinated possums, the apparent level of protection afforded by this route was less than by intranasal inoculation. This lack of substantial

correlation between LTA responses to PPD and protective immunity, may help explain why some possums which appear to be resistant to disease in the field, do not have a measurable LTA response (e.g. the case with the single gross inguinal lesion which had drained). Some possums which are infected with *M. bovis* via the respiratory route may possibly mount a successful local immune response only, without necessarily developing a peripheral blood LTA reactivity, if the disease does not progress.

There is evidence from infected possums without gross lesions, that some may have been infected for up to 5 years prior to necropsy (case 3747). In one case (2393) there is also evidence suggesting that lesions had been present, which resolved completely by the time of necropsy. Histological evidence from a small number of NGL animals, in which AFB have been found, would also suggest that of the nodes incorporated in the tissue pools for culture, both the axillary and the inguinal lymph node are likely to be the sites which contain the bacilli. In case 3747 the deep axillary nodes were also noted to have an unusual granular appearance, which although not thought significant at necropsy, may have been the site of infection.

These superficial lymph nodes are also predilection sites for gross lesions (Jackson *et al.*, 1995a), thus supporting the notion that they may also be the most likely sites for *M. bovis* isolation in the absence of gross pathology. As evidence suggests that these superficial lymph nodes are not commonly primary sites of infection, but are more likely to be secondarily infected following introduction of the organism to the lungs (Jackson *et al.*, 1995), it can therefore be assumed that the presence of *M. bovis* in these sites suggests the prior potential for lesion development at the site of initial entry. Thus NGL infected individuals have either been infected for a lengthy period and resolved primary lesions, or perhaps less likely, to never have developed visible lesions at the site of primary infection, but disseminated the bacilli haematogenously to the axillary and inguinal lymph nodes. In either of these scenarios, it would be impossible to estimate how long the bacilli may have been resident in the lymph nodes.

The relationship between the number of infected NGL to gross-lesioned possums (1:2), is similar to the situation observed in both red deer, ferrets and badgers (Gallagher *et al.*, 1976), where it is believed that approximately one third of all infected animals are without lesions at necropsy. This apparently constant ratio, implies the development of an equilibrium between various states of infection. This

suggests that in some cases the bacilli either die, decrease in abundance or become dormant, following a period of infection, such that culture of the predilection sites fails to isolate the organism.

It is well recognised that tuberculosis in humans, will after initial infection, remain quiescent, sometimes for many years, before, in some cases, a reactivation produces clinical disease (Blower *et al.*, 1995). This is often induced by a decrease in cell-mediated immunity, (Wiegeshaus *et al.*, 1989; Selwyn *et al.*, 1989; Das *et al.*, 1992). This may have been the case in some of the possums which showed early positive LTA reactivity, which later declined, with subsequent tests providing negative results prior to the animals dying from tuberculosis between 19 and 43 months following the initial positive LTA tests.

Resolution of even large fibrotic lesions has been shown to occur in a number of species (Gardner, 1922; Calmette, 1923; Rich and McCordock, 1929; Smith and Jones, 1961), but has perhaps been best characterised in humans with the assistance of radiography and adequate clinical histories (Rich, 1951). It is reasonable to assume that some lesions will resolve in this species also, perhaps better than in some others, such as pigs and cattle, as lesions in possums are neither characterised by extensive fibrosis nor calcification (Cooke *et al.*, 1995). The cases presented which demonstrate that lesion resolution in possums is possible, suggest that resolving lesions are characterised by low numbers of AFB, minimal fibrosis and granulomatous reaction (Figures 9-6 and 9-7). These findings are comparable with those in vaccinated possums challenged with virulent *M. bovis* (Aldwell *et al.*, 1995a; Aldwell *et al.*, 1995b). Superficial nodal lesions which discharge their necrotic contents, can apparently heal completely. The lungs of resistant animals, in which severe and extensive caseation was presumably present at some time, can conceivably undergo a process of resolution, whereby caseous material is gradually resorbed by the surrounding granulomatous reaction, thus resulting in the eventual reconstitution of the normal lung architecture (e.g. case 2191). There may be a future role for histopathological examination of frozen tuberculous tissues using histochemical staining techniques demonstrating enzymes such as β -galactosidase in macrophages. These methods have proven useful in laboratory animals for demonstrating the presence of competent activated macrophages (epithelioid cells) capable of destroying tubercle bacilli (Dannenberg, 1991), and have helped clarify

the role of macrophages in cell-mediated immune responses and the pathogenesis of tuberculosis.

Although culture was used as the definitive test for infection status of possums in these studies, this test procedure has its shortcomings. There is the possibility of cross-contamination occurring either in the field or in the laboratory, which could decrease the specificity of the procedure. However to our knowledge this was not a problem, as precautions were always taken to reduce the likelihood of cross-contamination. There may be more problems with false negative diagnoses, especially in NGL possums. In these cases pools of nodes were made from multiple sites, thus diluting the few bacilli which may be present on any one occasion, thereby reducing the chance of successful culture when the tissue sub-sample is placed into culture medium. Other procedures such as freezing and decontamination which were routinely employed, also reduce the viability of *M. bovis* (Comer *et al.*, 1995; G. de Lisle, pers. comm.). Dormant organisms or those with reduced viability, which have been apparently demonstrated in mouse models (McCune *et al.*, 1966a; McCune *et al.*, 1966b; de Wit *et al.*, 1995), may occur in other species. These mycobacteria fail to grow in artificial culture media, and their presence will reduce the sensitivity of cultural isolation. Notwithstanding these problems with cultural procedures, where appropriate samples from suspect lesions were submitted, isolation of the organism was achieved in nearly every case. Thus confidence in cultural procedures employed seems well deserved, but there will be an unknown proportion, presumably small, in which because of low numbers of bacilli (<100 per ml of tissue) (Butcher *et al.*, 1996) or presence of dormant forms, isolation attempts are unsuccessful.

If the organism is capable of dormancy in possums, the proportion of possums with dormant disease, which we will call "sleepers" (for lack of a more descriptive word), in the population will increase with as the prevalence of disease which can currently be diagnosed falls. This scenario could in part explain the difficulty in identifying tuberculous possums in cross-sectionally sampled populations in which there is only a low, and perhaps sporadic, incidence of disease. To evaluate the hypothesis of the occurrence of bacillary dormancy in possums it would be necessary to conduct a well planned field trial, in which *M. bovis* organisms could be induced to resume activity and produce disease, possibly by the administration of exogenous glucocorticoids. An alternative approach may be through the combined

cultural, and histological examination of axillary or inguinal lymph node tissues from possums with a high risk of possessing dormant organisms. Specialised histochemical techniques (Gutiérrez Cancela and García Marín, 1993; Perez *et al.*, 1996) or electron microscopy (Condrón *et al.*, 1994) which can detect the presence of mycobacterial antigen and cell wall-deficient L-forms (which are non-stainable with conventional techniques) may show the presence of mycobacteria in tissues from which *M. bovis* is not able to be isolated.

For those experienced with the appearance of gross lesions and the conduct of a detailed necropsy, as was the case with the people carrying out the necropsies in this study, the identification of tuberculous animals post-mortem was found to have a moderate sensitivity (0.83) and high specificity (1.0). The appearance of lesions are quite characteristic and rarely confused with other disease processes, especially in advanced cases (Cooke *et al.*, 1995), and single lesions in sites which may not be routinely examined are uncommon (Jackson *et al.*, 1995a).

Cases in which the diagnosis was made by histological examination alone, were based on finding of typical AFB in sections of tissues. Whilst these organisms may be other mycobacterial species, the recovery by culture of mycobacteria other than *M. bovis* is rare in possums. In the 370 cases subjected to mycobacterial culture in the longitudinal study there was only one isolate of *M. avium*, and this was recovered from a lymph node pool from a non-lesioned animal. Previously isolation of *M. avium* has been reported from one of 143 samples cultured in a series of cross-sectional studies reported by Jackson *et al.* (1995a), and also in one of 126 pooled lymph node samples cultured in 1986 (Livingstone, cited in Jackson, 1995a). These isolates of *M. avium* may possibly have been environmental contaminants of the submitted samples, rather than necessarily having caused infection in the possums from which they were taken. We can therefore be confident that the specificity of histopathology is very high. The same comment regarding the sensitivity of examination for tuberculous lesions is however not true. It has been estimated that as many as 1×10^4 organisms per ml of tissue need to be present before detection of AFB is likely (McCune *et al.*, 1966a). Possums without gross lesions are therefore much less likely to have microscopic lesions containing AFB detected, especially in large organs such as the lung, where it is impossible to examine the whole tissue (Jackson *et al.*, 1995a).

By comparison with diagnoses based upon combined LTA, ELISA and necropsy results, clinical examination for the diagnosis of tuberculosis was found to have a sensitivity of approximately 0.41. This figure was derived principally from multiple examinations of individual possums, so that this value represents the maximum sensitivity possible using palpation, as the sensitivity on any single occasion will be substantially less than this. The low sensitivity, apparently caused by numerous possums failing to develop a palpable peripheral lymphadenitis over the period they were under observation, or the lesions which did occur having discharged and healed over in between times of examination. The lower percentage of tuberculous possums identified by examination/necropsy compared with LTA in 1991/92 suggests that during a period when disease transmission may still have been active, that manual and cultural examination methods were able to identify only one fifth of individuals with evidence of exposure to *M. bovis* (assuming an 80% sensitivity of LTA).

The prevalence of tuberculosis on the Castlepoint study site, based on the number of possums known to be infected or having died from tuberculosis, peaked at approximately 20% in the first year, and subsequently declined over the ensuing few years (Jackson, 1995). The lowest prevalence was reached in 1993 when there was only one possum known to be infected on the site. At the end of 1993 the prevalence lifted as a series of new cases were discovered, most of which died after relatively short clinical phases. At the termination the prevalence was determined to be 6.3%, with most of the cases coming from the northern-eastern side of the site, and from possums which had not previously been captured. At this time it appeared there was, or had been, recent active spread occurring, as there were a number of animals with severe disease which were excreting *M. bovis* from the lungs and open fistulae, and two unusual cases of small single gross pulmonary lesions were found, which are probably indicative of recent acquisition of infection. The prevalence of positive LTA results for the years 1991/92 and after, shows the same trend observed in the prevalence of clinical disease in possums. The prevalence of LTA-positive possums in 1991/92 was 35%, and it could be speculated that if LTAs had been performed earlier in the study, that the prevalence of LTA-positive cases may have been substantially higher than found in 1991/92. It seems reasonable to assume that the estimates of infection prevalence from clinical observations were underestimates of the true prevalence of infection present on the site, because many possums were

in pre-clinical or latent stages of the disease at any given date. This being partly explained by one third of the apparently infected possums having no gross lesions, and consistently so over a wide range of gross disease prevalence (Table 9-XI).

This initial high prevalence, followed by a steady decline over a number of years, may be analogous to the situation seen at Flagstaff Flat on the West Coast (Coleman *et al.*, 1996). At this site the point prevalence was 60% in 1992. In annual cross-sectional studies since that date the point prevalence has fallen each year, such that in August 1996 the prevalence was less than 1% (J. Coleman, pers. comm.). If this represents the general pattern of disease prevalence in an undisturbed possum population, it suggests that a substantial population of “sleepers” and NGL possums may be periodically forced, by stressful environmental conditions, into a state of disease reactivation and bacillary excretion. These “sleepers” will thus potentially be the index cases which start a disease epidemic. The initial high to moderate incidence of recognisable disease in such an epidemic gradually declining over succeeding years as the proportion of resistant individuals increases, and the number of infectious possums falls. Although occurring on a different time scale, this is somewhat analogous to the transmission dynamics hypothesised for human tuberculosis epidemics (Blower *et al.*, 1995).

Of the tests evaluated none has shown itself to be useful for field application in cross-sectional studies. There is still substantial scope for development and improvement of serological tests, which if found to be sufficiently sensitive would have immediate field application. Lymphocyte transformation assay, whilst showing a moderate sensitivity, is handicapped by requiring careful blood collection to avoid clotting, and immediate dispatch to a laboratory equipped to perform the technically demanding and complex assay. Further evaluation of the LTA would be beneficial so that the attributes of the test, and lymphocyte responsiveness of diseased possums under natural conditions can be better understood. Testing for the presence of *M. bovis* DNA in blood may also provide another fertile area of diagnostic investigation (Barry *et al.*, 1993; van der Giessen *et al.*, 1994; Rolfs *et al.*, 1995).

The results of these investigations suggest that some possums can develop a degree of immunity to *M. bovis* infection, and amongst the population a range in susceptibility to the disease will be demonstrated. Some newly infected possums

will undergo direct disease progression and die within months of infection, whereas others may develop no disease whatsoever, and appear free of infection at necropsy. However many possums will become diseased, some becoming clinically apparent over varying time periods, and usually, but not invariably resulting in the death of the affected animal. A possum which survives infection may become a “sleeper”, and add to the reservoir of animals with dormant or quiescent infection, and in which lesions may be difficult to demonstrate. These latent cases may live for prolonged periods in a state of conditional immunity, supported by the presence of bacillary antigens, which continue to maintain a population of memory T cells. However, these possums may suffer from endogenous reactivation of disease induced by periods of stressful environmental conditions, and thus commence a wave of infection which will pass through a susceptible, pre-stressed population.

As with badgers (*Meles meles*), disease modellers may need to incorporate immune and latently infected classes into models if they are to be useful as predictive tools (Bentil and Murray, 1993; White and Harris, 1995). The apparent development of immunity in natural populations of possums also suggests that attempts now under way, at establishing individual immunity following vaccination of wild populations, are likely to be met with some success.

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The PhD candidate was responsible for managing the Castlepoint study site from July 1993 through till April 1996. Data collection and storage (with assistance) through this period, and subsequent analyses has been carried out by the candidate.

CHAPTER 10

ENVIRONMENTAL STRESSORS AND TUBERCULOSIS IN POSSUMS

Abstract

Possums (*Trichosurus vulpecula*) in New Zealand are maintenance hosts for bovine tuberculosis (*M. bovis* infection), and the presence of the disease in wild populations poses a serious threat to the success of eradication programmes for tuberculosis in domestic stock. Although the disease has been recognised in possums for 30 years the epidemiology in this species has only recently been clarified. A longitudinal study was established in 1989 to examine the disease behaviour in an infected possum population on a farm in the southern North Island of New Zealand, by trapping in a fixed set of 295 traps for 3 days per month. Animals captured were examined at 2 monthly intervals for evidence of tuberculosis. During the first 5.5 years of this project over 900 individual possums were captured and tagged. Blood was collected from each possum examined, and the sera retained was stored frozen.

Eight hundred and twenty-five sera from 341 possums were assayed for cortisol concentration, as it was hypothesised that the cortisol released as a response to the stress of a standardised trapping regime would provide a useful guide to the background stress levels to which the possums were exposed. The resulting data was examined by general linear modelling to show whether, climate, habitat, seasonal and possum characteristics had an influence on the apparent stress levels in the possums, and whether this in turn had a significant influence on the epidemiology of tuberculosis.

Variation in body weight was also analysed with general linear models, in a similar manner to serum cortisol data, as it was believed this would provide valuable insight into the nutritional status of possums and the effect that malnutrition may have on the epidemiology of tuberculosis. Additional investigations were also undertaken whereby serum cholinesterase, serum thyroxine, lymphocyte transformation assay and reproductive data were all used to investigate putative stressors. However, the results of these additional investigations were generally disappointing, and added little to understanding the effects of stress on possums.

Cortisol responses were shown to have a regular seasonal variation, with the highest peak response in the summer, and a lesser peak in mid-winter. The summer peak might be caused by heat, water or nutritional stress, and the winter peak by inclement weather or nutritional stress. These summer and winter cortisol peaks were apparently associated with a higher incidence of deaths from tuberculosis

during these periods. Females showed a minor elevation of stress responses around the two mating/birth periods in autumn and spring, but lactation was not associated with elevated stress responses. The highest cortisol levels occurred early in the study period, and were associated with low population density and low body weights, and preceded the most severe outbreak of disease. Possums which were known to have died from tuberculosis also showed higher cortisol stress responses than those with only lymphocyte transformation evidence of infection, or those showing no evidence of infection. This suggests that individual possum factors were influential in the progression of disease following initial infection. Although significant site effects were identified these were not associated with the prevalence of tuberculosis, which suggested that the clustered distribution of disease was dependent upon environmental factors other than the “stressful” nature of the habitat. Low average body weight of possums (a proxy for poor feed supply/drought conditions) also preceded outbreaks of disease. These outbreaks also appeared to be exacerbated by periods of wet, cool and variable weather occurring 1 to 2 months beforehand.

It was not possible to show a statistical association between the prevalence and incidence of tuberculosis and the weather. This arose as there are many other factors which will have an impact on the time from infection to clinical demonstration of disease and death. These factors will include the dose and route of infection, virulence of the organism and innate resistance of the host and individual circumstances which may alter the susceptibility of each possum. Given this set of complex factors, it was not surprising that the statistical methods used lacked the power to show a statistical association between weather patterns and the prevalence and incidence of tuberculosis.

The findings of this study suggest that glucocorticoid assays and monitoring of trends in body weight, are useful tools for investigating stressful environmental phenomena, and helpful in uncovering aspects of the epidemiology of tuberculosis in possums. Major stressful periods involving inadequate nutrition, heat, cold and moisture stress appear to precipitate severe tuberculosis outbreaks, which are believed to have their origins in the reactivation of subclinical/latent infection in the susceptible portion of the population. As the period of pre-clinical disease varies substantially, and can be as long as several years, this epidemic of tuberculosis takes

several years to subside. Thereafter a small number of clinically diseased possums are likely to be restricted to “hot spots” conducive to transmission of *M. bovis*.

Introduction

One of the hypotheses concerning the epidemiology of tuberculosis in animals and man, is that environmental stress plays a role in allowing full clinical disease expression, and may precipitate the death of infected individuals (Francis, 1958; Morris and Pfeiffer, 1995). There is general agreement that there is a complex interaction between the central nervous system, the immune system and the endocrine system, which when disturbed by stressors, orchestrates a response designed to maintain the homeostatic mechanisms of the individual (Bonneau *et al.*, 1990). These responses are generally beneficial to the organism in the short term, but may have detrimental effects if prolonged.

There are three major pathological consequences of chronic stress on an animal. The first of these is weight loss or reduced growth rate induced by catabolism of body tissues and loss of appetite, water loss, decreased fat deposition and alterations to protein metabolism. Chronic stress also inhibits gonadotrophin release, thus decreasing gonadal function, causing delayed puberty, lack of behavioural receptivity or libido, failure of ovulation and implantation or spermatogenesis and spontaneous abortion. Low birth weights, and decreased offspring survival have also been observed in stressed females (Kreeger, 1988; Johnson *et al.*, 1992). The other major effect is that of immunosuppression which is widely held to be due to the effects of increased circulating glucocorticoids (GC), high levels of which are known to impair macrophage function and destruction of mycobacteria (Schaffner, 1985; Cox *et al.*, 1989).

Experimental and clinical studies have demonstrated that stressful stimuli alter the activities of lymphocytes and macrophages in a complex way that depends upon the type of immune response, characteristics of the stressor, compartment of the immune system studied, and the timing of the stress relative to the immune response (Dantzer and Kelley, 1989; Lysle and Coussons-Read, 1995). A variety of chronic stressors have been shown to reduce delayed-type hypersensitivity (DTH) responses (Pitkin, 1965; Blecha *et al.*, 1982; Okimura *et al.*, 1986), and lymphocyte blastogenesis (Bell *et al.*, 1986; Rabin *et al.*, 1989; Niwano *et al.*, 1990; Becker and Misfeldt, 1995), predisposing affected individuals to development of tuberculosis following suppression of protective CMI responses.

In possums exposed to the stress of captivity, lymphocyte mitogenic responses were depressed, and a stress leucogram was found in the initial weeks following capture (Buddle *et al.*, 1992). Possums are known to be a corticosteroid-sensitive species (Khin Aye Than and McDonald, 1974), and stress of captivity has been implicated as a causal factor in acute salmonellosis, nematode problems, gastric ulceration and starvation in captive wild-caught possums (*Trichosurus vulpecula*) (Keber, 1979; Presidente, 1982). Severely stressed possums in poor condition have also been shown to have higher total and free circulating cortisol levels than animals in better condition, and this was associated with adrenocortical hyperplasia, focal necrosis and haemorrhage (Presidente and Correa, 1981). In an experimental *M. bovis* infection of three possums, Corner and Presidente (1981) found one possum showing adrenocortical hyperplasia, focal necrosis and ulceration of the gastric mucosa and ileal villus atrophy, to be the most severely affected animal by the infection. An earlier experimental study also implicated a stress-induced immunodeficient state, as a contributor to the rapid development of disease and death in *M. bovis* infected possums (Corner and Presidente, 1980).

Although evidence of factors initiating full clinical manifestation of tuberculosis in possums is limited, it would appear that stress factors, such as inclement weather which pushes possums outside their thermoneutral zone (van den Oord *et al.*, 1995), or imposes nutritional stress through shortage of suitable feed or difficulty in foraging through frequent rain, may be responsible. The aim of this study was to investigate the extent to which these putative environmental stressors may have affected the capacity of the immune systems of free-living possums at Castlepoint, to control infection with *M. bovis*. To this end, pre-existing data on weather, body weight, reproductive status and lymphocyte transformation assays, along with new measurements of circulating cortisol, thyroxine (T_4) and cholinesterase, performed on stored sera collected during the first phase of the "Castlepoint" study (Pfeiffer, 1994; Jackson, 1995) were examined.

With repeated stress there is adaptation of the hypothalamic-pituitary-adrenal (HPA) axis, resulting in attenuated responses to that stressor. Glucocorticoid and ACTH levels initially rise after the application of the stressor, but fall back to normal or near normal levels as the stress continues (Harbuz and Lightman, 1992). However, the habituation to a chronic stressful stimulus is stressor specific, such

that the application of a superimposed novel noxious stimulus will normally elicit an enhanced acute stress response, with higher and more persistent circulating GC levels than usual (Barret and Stockham, 1963; Sakellaris and Venikos-Danellis, 1975; Vernikos *et al.*, 1982; Jensen *et al.*, 1995; Hanlon *et al.*, 1995). This was the rationale on which the circulating cortisol measurements on possums were conducted, as it was believed that the stress imposed by a standardised cage trapping and examination regime would act as a novel stressor, which had the ability to amplify the prevailing background environmental stimulation of the adrenal cortex (Wingfield *et al.*, 1995).

Lymphocyte transformation assay (LTA) data was included in this investigation as the blastogenic response of mammalian lymphocytes to plant mitogens is believed to reflect the functional status of lymphocytes and the immune potential of the animal as a whole. The mitogenic response of lymphocytes is measured in terms of the incorporation of radio-active thymidine into DNA of the responding cells, since the amount of thymidine incorporated is known to reflect the strength of the response. Levels of mitogenic suppression have proved to be more sensitive indices of welfare than total lymphocyte counts, proportions of different lymphocytes, or delayed hypersensitivity (Broom and Johnson, 1993).

Circulating T_4 levels were measured in an attempt to determine if they could be useful indicators of the intensity of climatic or nutritional stress on the possums. Serum cholinesterase, a short half-life serum protein produced by the liver, has been used in human medicine as an indicator of short term malnutrition (Gofferje *et al.*, 1979; Ollenschläger *et al.*, 1989). In this study it was believed that investigation of cholinesterase levels may have given valuable insight into the nutritional status (i.e. gaining or losing weight) of possums, unavailable from body weight data.

Materials and Methods

A longitudinal study was established in 1989 to examine the disease behaviour in an infected possum population on a farm in the southern North Island of New Zealand, by trapping in a fixed set of 295 traps for 3 days per month (Pfeiffer, 1995). Animals captured were examined at 2 monthly intervals for evidence of tuberculosis. During the first 5.5 years of this project over 900 individual possums were captured and tagged. Blood was collected from each possum examined, and the sera retained was stored frozen.

Study site geography

For the purposes of categorisation for analyses, the study site covering 21 ha was broken down into four geographical areas. These divisions of the site were called the Northern, Southern and Middle sections, and Queen Street and these are depicted in Figure 10-1. A more comprehensive description of the site and its vegetation, including photographs are provided by Jackson (1995).

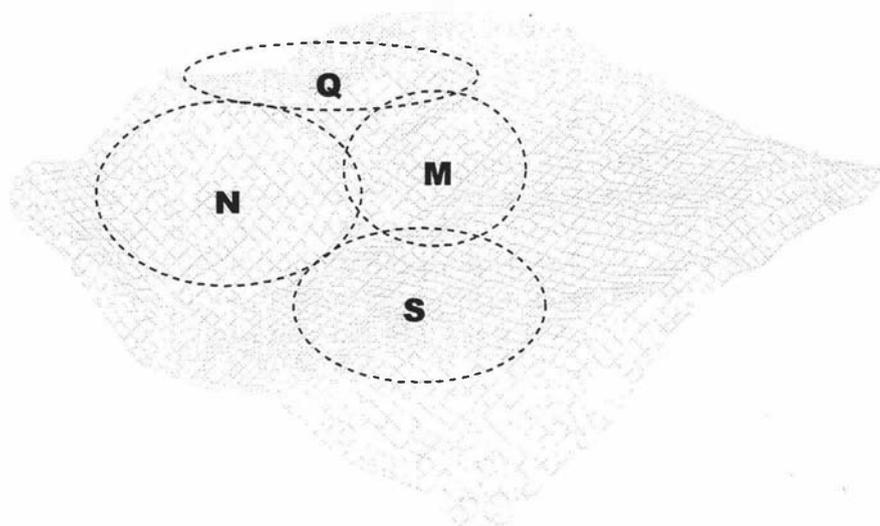


Figure 10-1. Digital terrain map showing the approximate spatial distribution of the four site categories used in analyses. S = southern section, N = northern section, M = middle section and Q = Queen street area

The Northern section was a south-facing hillside, which tended to be damp and was vegetated by mixed tree and shrub species on the lower slope, which were replaced by manuka (*Leptospermum scoparium*) and some gorse (*Ulex europaeus*) higher up. There was limited open pasture available on this site. The area was bordered to the north by Queen St, to the west by a gully with dense low forest and numerous tree ferns (*Cyathea* spp.), to the east by the Middle section, and to the south by the

Southern section. The northern section contained 78 trap sites and throughout the study 1316 examinations were carried out on possums caught in this area. The ratio of number of examinations to number of traps (a proxy for the possum density) was 16.87.

The Southern section lay on a northward facing slope, and was vegetated principally by scattered manuka and pasture. Part of the area was bordered by the Northern and Middle sections. This section held 84 trap sites, and accounted for 1144 possum examinations. The exam to trap ratio was 13.62.

The Middle section was mostly surrounded by the other sections, and contained the greatest mixture of plant species and ecotypes. The area was roughly sector shaped, the point of which ran down close to the valley floor, and the broadest side abutted Queen St. to the east and north. This section held 105 traps, accounted for 1747 possum examinations, the exam to trap ratio being 16.64.

Queen St. ran along an exposed gorse and manuka covered ridge which circled the eastern and northern sides of the study site and formed the boundary with the neighbour. Queen St. held 28 trap sites, which yielded 750 possums for examination, an exam to trap ratio of 26.79. On the western extremity of Queen St. there was a single long row of mature *Pinus radiata*. Other mature isolated pines were scattered through the Southern section of the study site, and one was found in the Middle section. A small pine plantation was also located adjacent to the southern most edge of the Southern section. Queen St. and the Middle section and the adjacent portion of Northern section, were the areas where the bulk of tuberculous possums were trapped throughout the study (Pfeiffer, 1994; Jackson, 1995). At the termination lesioned cases were restricted to Queen St. and the off-site area to the north of the Northern section.

Climatic data

Long-term weather data are recorded at Castlepoint, which is approximately 4.5 km to the south of the study site. Measurements of total monthly rainfall, average monthly temperature and ratio of average minimum to average maximum monthly temperature were taken from the records. This data had previously been used by Pfeiffer (1994) to calculate the long term monthly averages for rainfall and ratio of average minimum to average maximum monthly temperature. This data was used

to plot the actual monthly rainfall versus the long term average (Figure 10-2), and the actual ratio of average minimum to average maximum monthly temperature versus the long term average for this measurement (Figure 10-3).

The relationship between these plotted weather variables and the point prevalence and incidence of tuberculosis were examined statistically. The incidence, defined as the number of new cases divided by the number of possums examined at that visit, was examined with the use of logistic regression analysis using the statistical software package, Statistix for Windows (Analytical Software, Tallahassee, FL). Independent variables investigated included season, predicted body weight (from general linear model in Table 10-II) lagged by 1 month, whether the prior moving average for 2 months of total rainfall lagged by 1 month, was; 1) above the 75th or, 2) below the 25th percentile for that month, and whether the prior moving average for 2 months of the ratio of minimum to maximum monthly temperature lagged by 1 month, was; 1) above the 75th or, 2) below the 25th percentile. All main effects and second order interactions were screened in the model.

The adjusted point prevalence (as described and charted in Chapter 9), was examined by the general linear modelling procedure (GLM) of the statistical software package SPSS, version 7 (SPSS Inc., Chicago, IL). Independent variables screened in the model included the season, predicted body weight lagged by 1 month, 6 month prior moving average of the difference in total rainfall from the long term average for each month, lagged by 2 months, and the 2 month prior moving average of the difference in the ratio of the average minimum to average maximum monthly temperature from the long term average of the difference, lagged by 1 month, and first order interaction terms of the preceding variables.

Body weight and length

The body weight was recorded each time a possum was examined, as were tail and total body length. Examinations for each possum commenced when body weight was approximately 0.8 - 1.0 kg. The crude data for body length was modified such that when it was apparent that the possum had ceased growing, all subsequent lengths were standardised to a mean value after that date. Measurement error and degree of possum relaxation under sedation caused the length to vary by up to 6 cm between examinations. This data modification was felt necessary to improve precision of this parameter which was used as a covariate in a number of analyses.

Body weight was used as the dependent variable in two GLMs (Table 10-I) as it was believed that variation in body condition was likely to be a useful indicator of the nutritional status of the population, and a proxy for when animals were under physiological stress induced by malnutrition. The first analysis conducted involved no repeated measures on any individual, and was used to investigate the association between body weight and site, and to develop a predictive model (Table 10-II) which could be used to predict the weight of all possums examined, adjusted for the other parameters in the model. These predicted weights were used to depict the changes in body weight over time (Figures 10-6 and 10-7).

Serum collection

Sera assayed for cortisol and T_4 levels were principally collected from possums regularly cage trapped at the Castlepoint study site. Each possum was trapped in a cage set overnight. Prior to examination and measurement, which occurred at varying times of the day, each possum was tranquillised with an intramuscular injection of ketamine hydrochloride (40 mg/kg). Intra-cardiac blood collection was the last procedure performed during the examination. At the termination of the longitudinal study possums were anaesthetised with a mixture of ketamine hydrochloride (40 mg/kg) and diazepam (2 mg/kg) given by intramuscular injection prior to intra-cardiac blood collection and euthanasia. Sera were removed from clotted bloods after centrifugation, and stored temporarily at -20°C until they could be transported to Massey University, where they were then transferred to permanent storage at -80°C .

For the purpose of treatment comparisons, some possums were sampled after a period in captivity, or following soft-catch leg-hold trapping, and after shooting. The leg-hold trapped animals were sampled the morning following capture. Blood samples were collected into plain serum tubes as blood ran from the nasal cavity subsequent to lethal blow to the cranium. The other group were killed at night with the aid of a spot light, by a head shot using a 0.22 calibre rifle. Blood was collected by cardiac puncture as soon as the animal was retrieved, which was often up to three or four minutes after shooting. The small number of captive possums, which had been held for between 24 and 38 days at time of blood collection, had been used in calorimetry experiments (van den Oord *et al.*, 1995). Cardiac blood samples were collected under ketamine sedation from these animals immediately upon removal from the calorimeter in which they had been exposed to a temperature of 3°C for the

preceding 10 days. Blood samples were again collected from a subset of these same animals after 4 days 'recovery' period where they were held in individual cages inside a large indoor enclosure. The number of sera assayed for T_4 and cortisol concentrations for treatment group comparisons are shown in Table 10-V and Table 10-XI.

Delta Con A

For each of the lymphocyte transformation assays (LTAs) performed on possums (Chapter 9), and for which reliable results were available, the control (unstimulated) count was subtracted from the concanavalin A (Con A) stimulated count. This provided a new statistic, the delta Con A, which it was believed would furnish a useful index of the cell-mediated immune system's ability to respond to antigenic challenges. This data was collected over the interval from April 1991 up to and including the termination in August 1994, and involved 20 testing episodes. A square root transformation was applied to stabilise the variance of the observations. Due to the heterogeneity of delta Con A results between test episodes, the values for each episode were multiplied by a correction factor (range 0.94 - 2.28) which standardised the mean for each test episode, with respect to the results from the termination (correction factor of 1.0). This approach precluded the possibility of examining time related parameters in the data (Table 10-I). Where corresponding cortisol results were available ($n = 259$), a separate analysis, based upon previously conducted GLMs, was conducted to establish whether this parameter had any influence over delta Con A values.

Cholinesterase

Sera from 130 individual possums were subjected to cholinesterase assay. These were selected on the basis of having cortisol and delta Con A. results already available. The sera were assayed using a commercially available kit, Cholinesterase MPR3 (Boehringer Mannheim). Briefly, the assay entailed mixing 0.02 ml of sera with buffer/chromogen and substrate, and measuring the average change in absorbance over 30s in a spectrophotometer. The assay was performed in duplicate, and the resulting mean value was then multiplied by a temperature-dependent constant to derive the cholinesterase activity of the sera, measured in units per litre.

Thyroxine

Total serum T₄ assays were conducted with a commercial radioimmunoassay kit, Coat-A-Count Canine T₄, manufactured by Diagnostic Products Corporation, Los Angeles, CA. This assay utilises antibody coated tubes, and a canine serum matrix in the calibration solutions, which ranged in concentration from 0.25 to 15 µg/dl. The sensitivity of this assay is approximately 0.16 µg/dl. Each sample was tested in duplicate, and only results for which the coefficient of variation (CV) was <25% were used in the analyses and summary statistics. Radiation from the ¹²⁵I cortisol tracers was measured in a gamma counter (1261 Multigamma, LKB Wallac, Wallac Oy, Turku, Finland). The T₄ concentrations were determined with the aid of spline smoothed calibration curves using the programme Ria Calc, provided by the manufacturer of the gamma counter. All T₄ concentrations were assayed during the same test episode.

As it was believed that this product had not previously been used to assay possum sera, an investigation of its suitability for this species was conducted. A second set of calibrators was manufactured using charcoal stripped “blank” possum serum freed of thyroxine, and then combined with known amounts of T₄. These calibrators were used to produce another standard curve which was used to calculate an alternative set of assay results, for the assessment of whether there was a significant matrix effect. The procedure for removing T₄ from possum serum involved mixing a quantity of pooled serum with 1% Norrit-A activated charcoal, 0.1% dextran F70, and 0.2% sodium azide. This mixture was chilled overnight and centrifuged to remove the bulk of the carbon particles. The serum was then ultra-filtered through a millipore membrane to remove particles above a diameter of 0.2 microns.

Regression analysis was conducted on assay values derived from both the possum and canine matrix calibrators, and an equation developed which would allow the calculation of “true” hormone concentrations when using the results from canine matrix calibration. The reported results were derived from the use of canine calibrators. Thyroxine data from the various treatment groups was examined by the use of the Kruskal-Wallis one-way ANOVA on ranks to determine if there were differences in the median concentrations between groups.

Cortisol

Total serum cortisol assays were executed with a similar radioimmunoassay kit to that used for the T₄ assays (Coat-A-Count Cortisol, Diagnostic Products Corporation, Los Angeles, CA.) This assay utilised human serum matrix in the calibration solutions. Cortisol standards employed ranged from 0.5 to 20.0 µg/dl. The sensitivity of this assay was approximately 0.2 µg/dl. Gamma radiation measurement equipment used, and the method for calculation of results was the same as described for the T₄ assays.

Sera were tested in four time periods, using different batches of the radioimmunoassay kits. These episodes are hereafter referred to as Assays 1 to 4. Forty sera were tested in Assay 1, 224 in Assay 2, 420 in Assay 3, and 238 in Assay 4.

As it was also believed that this product had not been previously used to test possum sera, an investigation of its suitability for this species was conducted. During Assay 2, another set of calibrators was manufactured using the “blank” possum serum and following similar procedures to the T₄ assays. These possum serum calibrators were also used to assess whether there was a significant matrix effect on the assay results. Intra-assay cortisol variation was assessed during Assay 3, wherein 13 samples were tested in quadruplicate.

As there were large interassay variations (between batches/test episodes) in cortisol values it was found necessary to adjust the transformed results from each test episode so that these could be combined for analytical purposes. The results of Assay 2 were used as the referent to which the results of other test episodes were compared. Repeated measures analysis of variance (ANOVA) was employed to help assess which test episodes were significantly different, and the ratio of the mean value of each test comparison used to adjust the cortisol values. Not all of the same samples were retested on each occasion. Regression analysis, examining the relationship between assay values derived from both the possum and human matrix calibrators was conducted, and an equation developed which would allow the calculation of “true” hormone concentrations when using the results from human serum calibration. The results reported are those derived from the human matrix calibrators.

The cortisol concentrations for the shot, leg-hold trapped, and the cage trapped possums was subjected to the Kruskal-Wallis one-way ANOVA on ranks to establish whether there were differences in the median concentrations between the three groups.

Statistical Analysis

General linear modelling

As most of the dependent variables of interest in this study were continuous in nature, general linear modelling was used to identify factors which significantly influenced the dependent variables. The analyses followed standard procedures. All independent variables were first screened as main effects only. Those approaching the 0.10 level of significance were retained for further investigation, which involved the examination of all first order interactions. Factors were eliminated from the analysis in a step-wise fashion till only those significant at the 0.05 level remained in the final model. Where appropriate, contrasts were used to identify which categories of a factor were significantly ($p < 0.05$) different from each other.

All statistical analyses were performed using the software package SPSS, version 7 (SPSS Inc., Chicago, IL). A summary of the parameters (including numbers and descriptions) initially investigated in the various linear models is presented in Table 10-I.

Table 10-I. List of analyses undertaken using the general linear modelling procedure of SPSS

Dependent variable	N	Fixed factors	Covariates	Results table
weight	5017 ^r	sex, site, month, quarter	lnage, length ³ lnage*length ³ , sex*lnage	Figure 10-6, Figure 10-7
weight	959	sex, site, month, cod, quarter, quarter*month, site*cod	lnage, length ³ , lnage*length ³ , length ³ *sex, lnage*sex	Table 10-II
cortisol ^{0.5}	825 ^r	sex, site, haemolysis, month, quarter, sex*site, month*quarter, month*site, tb death,	lnage, length ³ , length ³ *weight, caught ^{0.25} , serum age	Table 10-VI
cortisol ^{0.5}	340	sex, site, haemolysis, season, season*site, tb death, sex*site	lnage, length ³ , length ³ *weight, caught ^{0.25} , serum age	Table 10-XVII
cortisol ^{0.5} (females only)	383 ^r	site, month, quarter, lactation, month*quarter	length ³ , weight, caught ^{0.25} , length ³ *weight	Table 10-IX
cortisol ^{0.5}	158	tb status, sex, site, season- (winter and spring only),	caught ^{0.25} , lnage, length ³ , weight	Table 10-X

Table 10-I continued

Dependent variable	N	Fixed factors	Covariates	Results table
deltaConA ^{0.5}	744 ^r	sex, site, tb death	lnage, length ³ , length ³ *weight, caught ^{0.25} , lnage*weight, length ³ *lnage, site*weight	Table 10-XVI
deltaConA ^{0.5}	321	sex, site, tb death	lnage, length ³ , caught ^{0.25} , lnage*caught ^{0.25} , length ³ *lnage,	Table 10-XVII
CHE ^{0.5}	118	sex, site, haemolysis, month, quarter, tb death,	lnage, length ³ , length ³ *weight, , serum age, caught ^{0.25} , deltaConA ^{0.5} , cortisol ^{0.5}	NA
T ₄ ^{0.5}	161 ^r	sex, site, season, haemolysis	length ³ , weight, caught ^{0.25} , length ³ *weight, lnage, serum age, cortisol ^{0.5}	Table 10-XII
T ₄ ^{0.5}	93	sex, site, season, haemolysis	length ³ , weight, caught ^{0.25} , length ³ *weight, lnage, serum age, cortisol ^{0.5}	Table 10-XIII

Key to terms used in table:

cortisol^{0.5} = corrected cortisol values, square root transformed

T₄^{0.5} = thyroxine values, square root transformed

CHE^{0.5} = cholinesterase values, square root transformed

deltaConA^{0.5} = delta Concanavalin A values, square root transformed

r = repeated measures on individual possums included

site = section of the study site in which the possum was trapped

season= winter (June through August), spring (September through November), summer (December through February), and autumn (March through May)

quarter = longitudinal study time periods divided into 23 consecutive year quarters corresponding to seasons of the year. Quarter 1 includes only April and May 1989, and quarter 23 includes September and October 1994

tb death = whether possums were known to have died from tuberculosis or not

haemolysis = degree of haemolysis evident in serum sample. Visually scored, with values of 0, 1, 2 or 3

tb status = possums categorised 3 ways depending upon tuberculosis diagnostic status (see details in text)

cod = cause of death, 4 categories i.e. exposure starvation, killed (or accidental death), unknown or disappeared, and tuberculosis deaths

lactation = 3 categories for females i.e. not lactating; pouch young estimated to be less than 80 days of age; and pouch young over 80 days old.

serum age = time in days, from when the sample was collected

caught^{0.25} = number of times an animal was captured, transformed to the power 0.25

lnage = natural logarithm of possums estimated age in days (see Appendix IV)

weight = possum liveweight in kilograms

length³ = total adjusted possum length in centimetres, transformed to the power 3

Cortisol levels and tuberculosis status

A data set was constructed to allow examination of the effect of tuberculosis status on the adjusted and transformed cortisol values. Possums were categorised into three groups. These were: 1) Those which showed no evidence, from clinical examination, LTA, or necropsy, of ever having had *M. bovis* infection. 2) Those which were LTA positive (Chapter 9) at some time but which had no other evidence or suspicion of infection. 3) Those which were known to have died from *M. bovis* infection, or would have died if not killed for some other reason. Sera from the tuberculous possums in advanced or terminal stage disease were avoided, and results of cortisol assays and the site of capture, were disregarded in the sample selection process.

The set of sera chosen for the assays was composed only of those from possums which had been cage trapped, and was balanced for season of collection, such that approximately equal numbers of each sex, in each of the three disease groups, were taken from either winter or spring (the only seasons included in the sampling). The final data set had the following composition: Groups 1: 28 females and 29 males, Group 2: 21 females and 29 males Group3: 21 females and 30 males. There were no repeated measurements on any single possum.

From each of the above three categories of possums, nine animals were selected on which to perform a series of serum cortisol measurements. The number of sera assayed for each possum varied between 9 and 24, with an average of 12.4 measurements per possum. The time period and duration, over which the measured sera were collected varied for each possum, but commenced at or soon after first capture, and finished with the last sample collected prior to death or disappearance. The data were plotted to see if there was any apparent pattern which may have related to tuberculosis status, or stage of disease to trends in cortisol levels. Coefficients of variation were calculated for the cortisol values for each of the 27 possums, and the variance of the CVs for the three categories examined using the Kruskal-Wallis procedure.

Reproductive parameters

One-way analysis of variance, was used to examine the relationship between year (1989 - 1994) and the mean date of birth of offspring born in the autumn birth season. Birth dates of pouch young were determined as described in appendix IV.

The number of days from the 1st of April till the estimated date of birth was employed as the dependent variable. The number of offspring born to immature mothers (with estimated ages between 1 and 2 years), each year, was also determined to assess whether there may have been some relationship between putative stressors and delayed puberty.

Survival analysis, as described in Chapter 9 was used to test for differences in lifespan of tuberculous male and female possums, as it has been suggested that the lactation period may be stressful to females and predispose to development of tuberculosis (D. Pfeiffer pers. comm.). Analyses were conducted separately for those possums which were found tuberculous by any of culture, histopathology, necropsy or clinical examination (n = 110), and those which had evidence of infection, but only from blood tests (n = 78) (see Chapter 9).

Results

Climatic effects

The results of logistic regression analysis of meteorological factors which may have been influential on the incidence of disease failed to produce a model in which any confidence could be placed. A similar outcome resulted from the attempt to model factors which may have been associated with point prevalence of infection. However, examination of the charts showing the variation in total monthly rainfall versus the long term (1972-1990) average monthly rainfall (Figure 10-2), the average ratio of monthly minimum to maximum temperature versus the long term average (Figure 10-3), the incidence of new cases (Figure 10-4) and the point prevalence of disease (Figure 9-1, Chapter 9), suggests that there may be some biologically plausible associations between climatic events and the pattern of disease. Anecdotal evidence suggested that the start of the longitudinal study was preceded by a period of drought. This is corroborated by the rainfall data (Figure 10-2), although several important data points shortly prior to Visit 1 were unavailable. This dry period immediately preceded a wet, cool and variable autumn and winter, during which the incidence of tuberculosis climbed rapidly from a low base. This was followed by a period of several years when the weather remained mild and stable, around the long term averages. The 'adjusted' prevalence during this period initially remained high but gradually declined from the peak observed 5 months into the study. A second peak in the prevalence occurred following another dry period which included a cool and variable winter and spring in 1993.

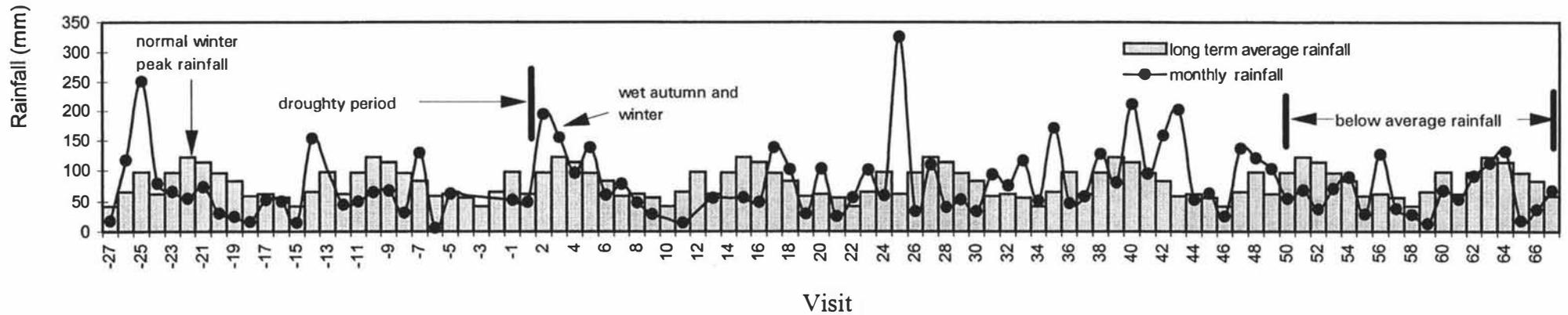


Figure 10-2. Average monthly rainfall data, commencing 27 months before the start of the longitudinal study. The long term monthly average (1972 - 1990) is also shown for comparison (Pfeiffer, 1994). Some points missing as data unavailable

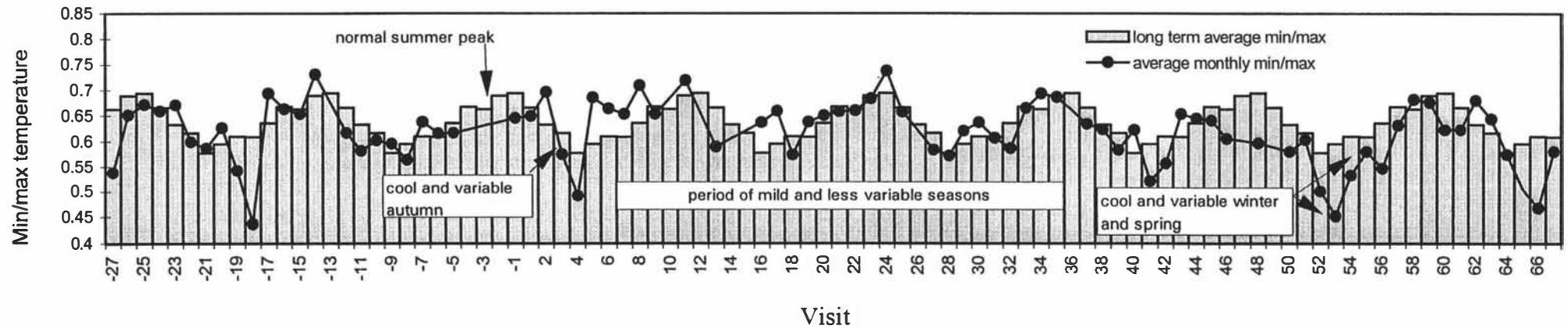


Figure 10-3. Average monthly minimum temperature (°C), divided by the average monthly maximum. Long term average figures are presented for comparative purposes (Pfeiffer, 1994). The value falls as variation increases and average temperature declines

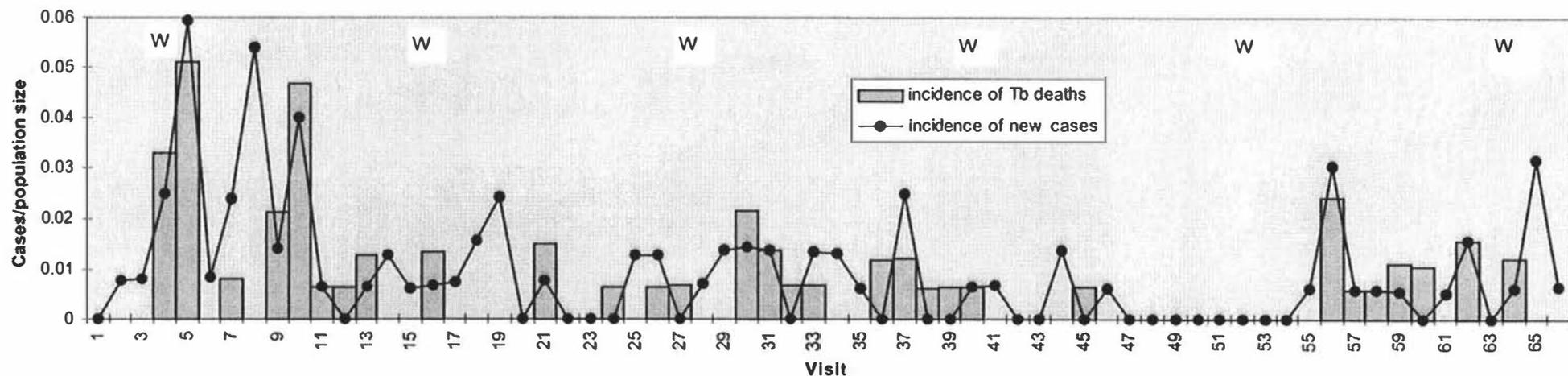


Figure 10-4. Plots of the incidence of new tuberculosis cases and deaths due to tuberculosis in possums at the Castlepoint study site. “W” denotes the mid-winter month of July

The denominator used to calculate the incidence figures were those taken from the Jolly-Seber population estimates derived from the computer software package “Caro” (Pledger and Efford, 1997) (see appendix II). The incidence values for new cases included those possums detected by clinical examination, or necropsy following natural death or after killing. Incidence of deaths attributable to tuberculosis was established by the examination of carcasses following natural death. There is no clear seasonal pattern in either tuberculous deaths or in new tuberculosis cases evident in the data. However, plots of incidence of new cases and deaths by month (Figure 10-5) shows that the number of deaths due to tuberculosis peaks in both mid-winter and summer, and corresponds to the periods of peak cortisol concentrations (Figure 10-9), whereas the incidence of new cases is rather variable but shows a tendency for more clinical cases to become apparent in the late winter-spring.

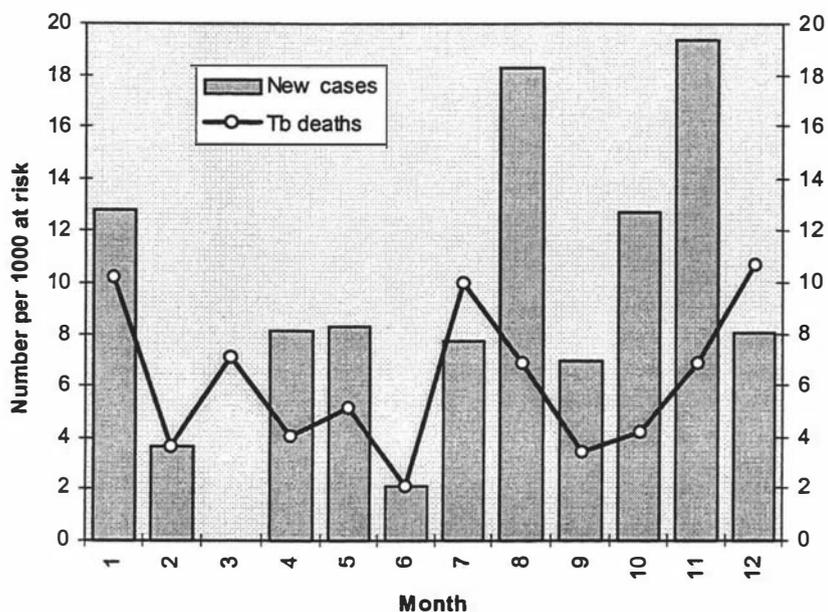


Figure 10-5. Standardised incidence of newly diagnosed tuberculosis cases and deaths due to tuberculosis, by month of the year

Body weight

The factors which were found to have a significant effect on the body weight of possums are presented in Table 10-II. The same linear model was applied to the full data set of all possum examinations, which included 4987 records from 945 possums. This analysis was used to generate predicted weight values which were used to plot the variation in body weight over the duration of the longitudinal study (Figure 10-6) and over an annual cycle (Figure 10-7).

Table 10-II. General linear model examining the effect of sex, site, month, quarter, length and age on the weight of possums. N = 945 (no repeated measures)

Model variables	DF	F - value	p - value	Eta squared ^a
intercept	1	0.3	0.596	<0.01
sex	1	20.7	<0.001	0.22
site	3	9.9	<0.001	0.03
month	10	4.0	<0.001	0.04
quarter	21	3.8	<0.001	0.08
length ³	1	97.3	<0.001	0.10
lnage	1	7.0	0.008	0.01
length ³ *lnage	1	45.2	<0.001	0.05
sex*lnage	1	25.2	<0.001	0.03

Adjusted R² = 0.85

a estimate of the variance explained by term in the model

The estimated marginal means for body weights (kg) at the four sites, middle, Queen St, north and south are presented in Table 10-III. The possums examined from the middle section and Queen St. had significantly heavier weights than those examined at other sites.

Table 10-III. Estimated least squares mean body weight by site. Weights with the same superscript symbol are significantly different from each other

Site	Mean body weight (kg)	SE
Middle	2.082 ^{**†}	0.014
Queen St.	2.041 ^{§†}	0.016
South	1.999 ^{*†}	0.015
North	1.987 ^{†§}	0.015

Figure 10-6 shows that initial weights during the study were low but rose steadily for the first 2 years, then gradually declined for the next 3 years, and fell precipitously in the final year. The change in body weight with each month in the year is shown in Figure 10-7. The maximum weight is reached in February, and the minimum weights persist through from May to September, apart from a transient increase in August. This August weight gain was a prominent feature in years 1, 3, 5 and 6 of the study, but was not apparent in years 2 and 4 (data not shown). There is a period of rapid weight increase in the period from December to January. Males have heavier weights than females but the annual cycle of variation in weight is similar for both sexes.

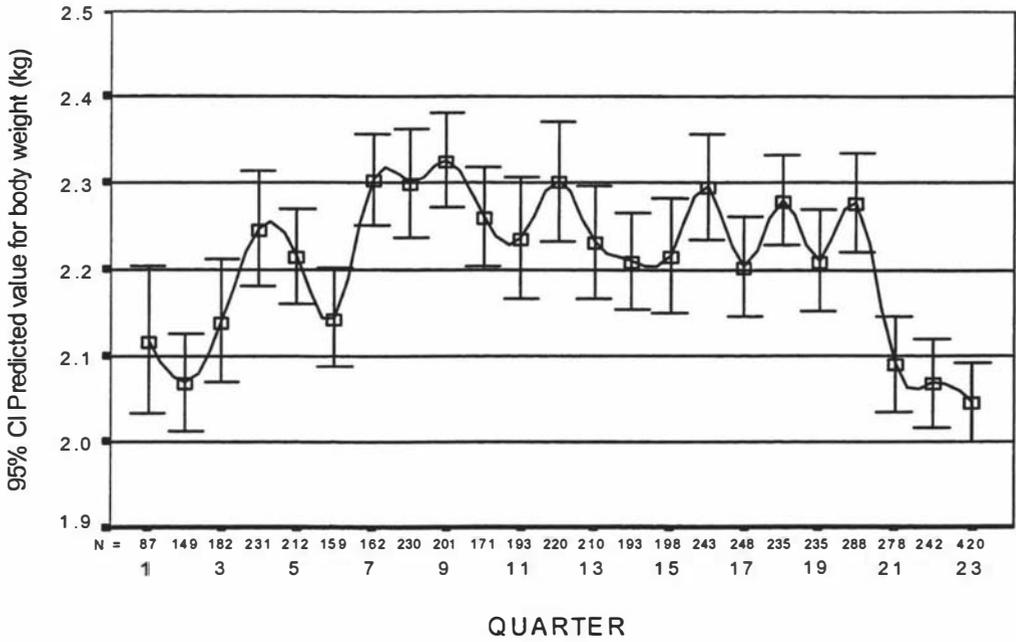


Figure 10-6. Plot of average body weight of possums throughout the longitudinal study, adjusted for significant parameters shown in Table 10-II

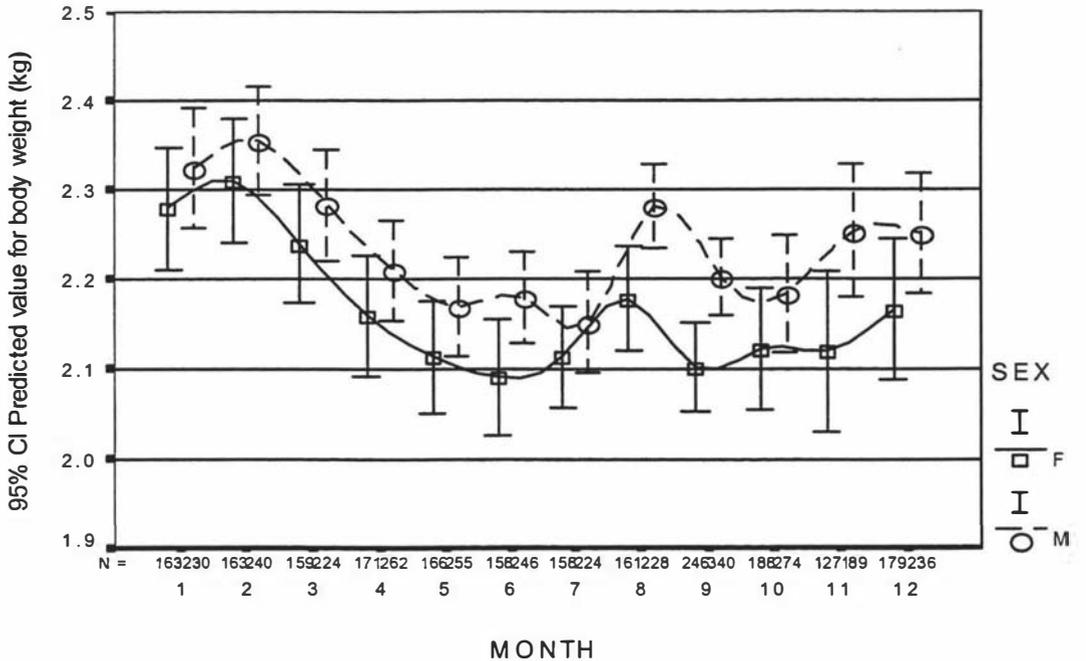


Figure 10-7. Plot of average body weight (males and females) for each month of the year, adjusted for significant parameters shown in Table 10-II

Reproductive performance

Examination of the mean time of birth in possums during the autumn seasonal birth peak is shown in Table 10-IV. The mean date of birth followed the same pattern as the annual body weight changes, in so far as low weights were associated with slightly delayed breeding. The use of contrasts showed that the mean birth date in 1994 was significantly later than each of the other birth dates. This delay coincided with a precipitous fall in mean body weight in the possums. The mean in 1989 was not significantly different from any other year. This suggests that the rapid weight loss observed in 1994 delayed the breeding of possums in that year, but that low body weight in the first year apparently had little effect, or the number of offspring in that year was insufficient to allow a significant difference to be demonstrated.

The number of immature females, with estimated ages between one and two years, which gave birth in each calendar year was also investigated. The results for the years from 1989 through to 1994 were 24/50 (0.48), 18/45 (0.40), 9/29 (0.31), 18/32 (0.56), 21/35 (0.60), 26/63 (0.41) respectively. The results for the 1994 season may be a slight underestimate as all possums were killed before the completion of the spring breeding season. No pattern in the data was discernible which could be related to climatic variables, body weight or incidence of tuberculosis.

Table 10-IV. Results of ANOVA investigating the effect of year of birth on the number of days from the 1st of April till the mean birth date of offspring born in the Autumn birth season (F = 4.09, df = 5, p = 0.001)

Year	N	Mean (days)	95%CI	Range (days)
1989	47	30.15	25.46 - 34.85	2.2 - 68.7
1990	51	29.22	24.65 - 33.80	6.3 - 75.9
1991	43	26.08	20.95 - 31.21	2.5 - 74.2
1992	42	24.27	19.20 - 29.35	4.9 - 62.6
1993	67	31.80	26.90 - 36.70	-3.1 - 83.4
1994	73	37.87	33.25 - 42.50	7.9 - 88.5

Kaplan-Meier survival analysis for the confirmed tuberculous possums found the mean and median age at death for females were 50 (41-58) and 44 (34-54) months respectively, and for males 43 (36-50) and 38 (31-45) months respectively. The logrank test over all pairs pooled showed that the two curves were not significantly different from each other, having a χ^2 statistic of 1.64 (p = 0.20).

For those possums which were diagnosed tuberculous on the basis of a positive blood test, the mean and median survival periods for females were 73 (58-87) and 88 (53-123) months respectively, and for males 80 (63-97) and 83 (37-129) months respectively. The logrank test over all pairs pooled showed that the two curves were not significantly different from each other having a χ^2 statistic of 0.0 ($p = 0.95$). Taken together, these results suggest that for infected or possibly infected possums, there is no relationship between sex and longevity.

Cortisol

The mean intra-assay coefficient of variation was found to be 14.1% (range 2.6% to 31.1%). The final estimate of the interassay coefficient of variation was 23.7%. Parallelism in the cortisol assay was investigated by the serial dilution of 3 samples with "blank" possum serum. The response to dilution showed that there were non-specific inhibitors of binding present in the possum serum matrix which will have introduced systematic errors to the measurement of cortisol concentration. At expected concentrations of $\geq 1.5 \mu\text{g/dl}$ interference was negligible, but rose in significance at lower concentrations, such that at expected concentrations of $0.35 \mu\text{g/dl}$ the percentage of observed/expected was 51%.

The results of the simple linear regression of cortisol concentration (square root transformed) derived from possum matrix calibrators (dependent variable) and the concentration determined with human calibrators, using 141 data points and with the line forced through the origin, produced a regression coefficient of 1.118 (95% CI = 1.080 - 1.116), with an adjusted R^2 of 0.96.

Repeated measures ANOVA, applied to 12 samples tested in both Assay 2 and 3, showed the presence of a significant and consistent difference between the two results ($p = 0.004$). Assay 3 results were adjusted by a factor of 1.097, this being the ratio of the mean cortisol value of the 12 samples tested in both assays. Assay 1 had four samples which were retested in Assay 2, and although not found to be significantly different ($p = 0.11$), the direction of the difference was consistent among the 4 samples. An adjustment factor of 0.913 was applied to Assay 1 results on the assumption that the observed difference was real. Eight samples were retested in Assay 4, and the difference between this assay and Assay 1 was neither significant ($p = 0.73$) nor consistent between assays, thus no weighting factor was applied to these results.

Treatment effects

Serum cortisol concentrations ($\mu\text{g/dl}$) derived from the human matrix calibrators, are summarised with respect to possum treatment category in Table 10-V. Back transformation from the square root transformed data has been used where necessary. One high outlier was removed from among the data for the shot possums, as it was likely that there was considerable delay from the time the possum was first shot, till death and sampling. All data represents independent observations within each population sampled. Data for the three classes with over 13 observations, was distributed in a log-normal manner.

Table 10-V. Summary statistics on serum cortisol levels ($\mu\text{g/dl}$) from possums subjected to a variety of treatments

Sampled population	N	Mean	Median	SD	Range
Cage trapped	340	3.39	3.14	1.83	0.14 - 11.33
Leg-hold trapped	14	3.37	3.29	2.22	0.65 - 9.88
Shot	25	0.69	0.42	0.69	0.09 - 2.91
Calorimetry 1 ^a	5	1.12	0.87	1.09	0.14 - 2.91
Calorimetry 2 ^b	3	1.47	1.50	1.15	0.30 - 2.60

a blood collected immediately after removal from calorimeter

b blood sample taken after 4 days recovery in indoor cages

The analytical results showed that at least one median (not including calorimetry possums) was different from the other two (corrected $\chi^2 = 54.2$, $p < 0.001$). The Kruskal-Wallis multiple-comparison Z-value test was used to determine which of the medians differed. The results showed that there was no significant difference between the leg-hold and the cage trapped animals (z-value = 0.30), whereas the shot possums were significantly lower than both the leg-hold and cage trapped possums (respective z-values: 4.33 and 7.36).

The results of the GLM analysis shown in Table 10-VI, suggest that section of the study site has an influence on the stress levels on possums which live mostly in that area. The estimated marginal means for the Middle, Queen St., South, and Northern sites were, 1.64, 1.69, 1.76 and 1.77 respectively. Although site effects were significant, contrasts could only be successfully used to examine the effects on cortisol levels in a simpler model which did not include site interaction terms. Contrasts applied to a simpler model showed that the Middle section cortisol levels were significantly different from the Northern and Southern sections, suggesting that that the Middle site (and possibly Queen St.) provided a less stressful environment than the other sections of the study site.

Table 10-VI. Model of significant factors which influenced the cortisol level (square root transformed) of possums on the Castlepoint study site. This includes 825 observations on 341 possums

Model variables	DF	F - value	p - value	Eta squared
intercept	1	4.3	0.038	0.006
sex	1	96.6	<0.001	0.119
site	3	2.9	0.031	0.012
month	8	1.8	0.067	0.020
quarter	19	2.0	0.007	0.050
caught ^{0.25}	1	26.8	<0.001	0.036
weight	1	6.3	0.012	0.009
length ³	1	15.1	<0.001	0.021
month*quarter	36	1.7	0.007	0.079
length ³ *weight	1	9.9	0.002	0.014
month*site	33	1.7	0.011	0.072

Adjusted R² = 0.32

Parameter estimates for length³ and weight showed that they were positively associated with cortisol levels, although the magnitude of the effect suggested only marginal importance.

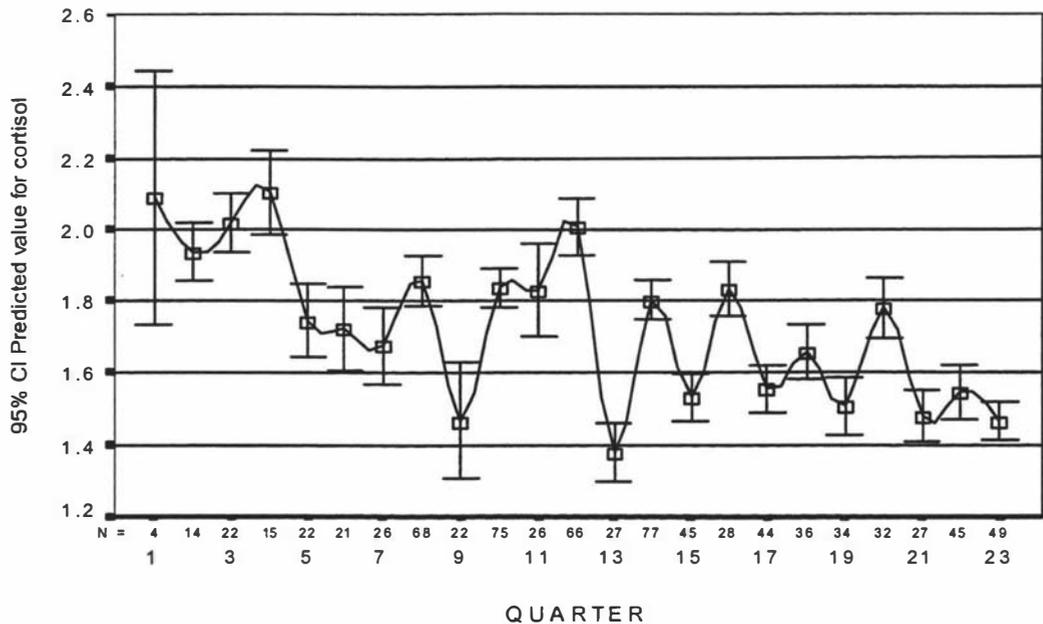


Figure 10-8. Plot of the cortisol^{0.5} levels predicted by the general linear model shown in Table 10-VI, versus time (quarter). High peaks occurred each summer

A plot of the variation of cortisol levels over the duration of the longitudinal study (Figure 10-8), shows that cortisol levels were higher around the commencement of the study and gradually declined thereafter. Cortisol levels also peaked each summer throughout the six years.

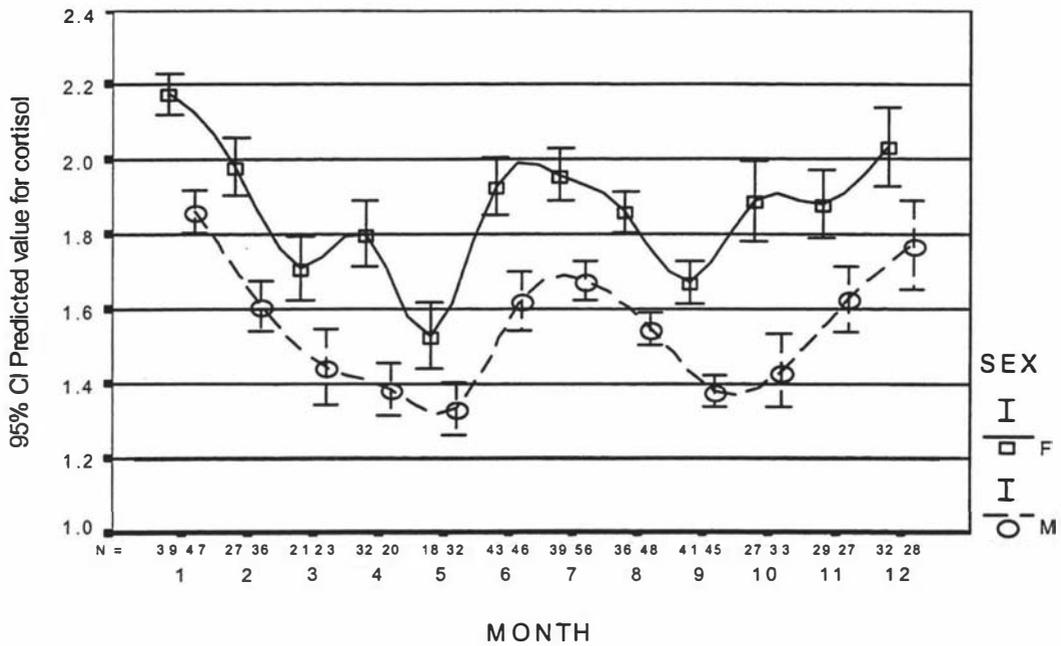


Figure 10-9. Plot of the cortisol^{0.5} levels predicted by the model shown in Table 10-VI, showing the variation in both sexes over the twelve months of the year

The cortisol levels appear to follow a regular annual cycle (Figure 10-9) with summer and winter seasonal peaks, which are highest in the summer. This cycle is similar for both sexes although the males are apparently less stress responsive than females. Low stress periods seem to be in both autumn and spring, although the autumn and spring troughs for females are interrupted by apparently stressful periods in April and October which produced a small rise in the cortisol levels for those months.

When the cortisol data was examined without the use of repeated measurements on any possum, sex, season and number of times captured were the only variables which were found to have a significant effect on cortisol levels (Table 10-VII). The lower power of this analysis precluded the inclusion of several factors which were found to have minimal influence in the analysis which included repeated measures.

Table 10-VII. Model showing the effect of sex, season and number of times captured on the cortisol level (square root transformed) of possums on the Castlepoint study site. (n = 340, without repeated measures on any individual)

Model variables	DF	F - value	p - value	Eta squared
intercept	1	729..7	<0.001	0.686
sex	1	47.6	<0.001	0.125
season	3	3.8	0.010	0.033
caught ^{0.25}	1	26.5	<0.001	0.074

Adjusted R² = 0.19

Contrast use showed that the seasonal effects found in the more comprehensive analysis were also present in this examination of the data (Table 10-VIII). Summer and winter were the seasons associated with a higher stress response in possums.

Table 10-VIII. Estimated marginal mean serum cortisol concentrations for the four sections of the study site. Cortisol values with the same superscript symbol are significantly different from each other

Season	Mean cortisol ^{0.5}	SE
Summer	1.891 ^{§†}	0.055
Autumn	1.638 ^{§†}	0.070
Winter	1.856 [†]	0.040
Spring	1.741 [†]	0.051

The lactational effects on cortisol stress responses are presented in Table 10-IX. The results show that the stage of lactation has a significant effect on cortisol levels when a model incorporating repeated measures was used. The estimated marginal means for non-lactating females, those estimated to have pouch young up to 80 days of age, and those with pouch young over 80 days old were 1.91, 1.93 and 1.79 respectively. Contrasts showed that only the cortisol levels between early and late lactation were significantly different. Stage of lactation was not found to be a significant factor when the female data was examined without the inclusion of repeated measures.

Table 10-IX. Final model examining the effect of lactation, quarter, length, weight, and number of times captured on cortisol levels in female possums. Based upon 383 records from 131 females, of which 96 were lactating

Model variables	DF	F - value	p - value	Eta squared
intercept	1	0.09	0.768	<0.00
lactation	2	4.2	0.015	0.02
quarter	22	3.5	<0.001	0.18
length ³	1	20.1	<0.001	0.05
weight	1	13.2	<0.001	0.04
caught ^{0.25}	1	5.3	0.022	0.02
length ³ *weight	1	16.8	<0.001	0.05

Adjusted R² = 0.21

Effect of tuberculosis status on cortisol level

The final GLM is presented in Table 10-X, and shows that there was a significant association between tuberculosis status and cortisol levels ($p = 0.019$). The means for each of the disease status groups, 1, 2, and 3, were 1.61, 1.62 and 1.85 respectively. By using contrasts, the mean of group 3, i.e. those which died from tuberculosis, were shown to be significantly different from the other two combined and from each group independently. Groups 1 and 2, i.e. those which had no evidence of tuberculosis or only LTA evidence, were not significantly different from each other.

Table 10-X. Model investigating the effect of tuberculosis status, sex and the number of times captured on blood cortisol level in 158 possums

Model variables	DF	F - value	p - value	Eta squared
intercept	1	220.1	<0.001	0.59
Tb status	2	4.0	0.019	0.05
sex	1	19.5	<0.001	0.11
caught ^{0.25}	1	9.5	0.002	0.06

Adjusted R² = 0.20

Examination of the results of serial cortisol measurements on 27 selected possums found no apparent patterns in the plots of cortisol concentration over time, which could be related to clinical status. There was no suggestion from the data to implicate tuberculosis infection, at any stage of the disease, in causing either an elevated or diminished cortisol stress response. However, there was evidence suggesting that 13 possums (48.1%) were consistent in their stress response,

whether high, low or intermediate, as the coefficient of variation (CV) in serum cortisol levels was less than 20% (median 21.8%, range 8.5 - 36.9%), whereas the remainder were more variable in their stress responses. There was a suggestion from the data that those possums with only LTA evidence of infection were less variable in their cortisol responses (mean CV = 18.8%) than those with no evidence of infection (mean CV = 24.7%), or those which died from tuberculosis (mean CV = 24.3%). However, comparison of the coefficients of variation of the three categories using the non-parametric Kruskal-Wallis one-way ANOVA on ranks failed to show any significant difference between the CVs of three categories of possums ($p = 0.30$).

Thyroxine

Treatment effects

Intra-assay variation was calculated for the T_4 assay by obtaining the mean coefficient of variation of 121 duplicate samples for which the assay produced a meaningful result. The resultant mean CV was 21.3%, a high figure attributed to the sera having T_4 concentrations down in the low end of the assay, with some being below the sensitivity of the assay. As many low serum T_4 values were discarded through having high CVs, the summary results presented in Table 10-XI are believed to be somewhat biased, so that the medians and means presented will actually appear higher than was actually the case. Distribution of the results for the cage trapped animals was log-normal, and the other categories contained too few samples to determine the underlying distribution.

Table 10-XI. Summary statistics on serum T_4 levels ($\mu\text{g/dl}$) from possums subjected to a variety of treatments (no repeated measurements)

Sampled population	N	Mean	Median	SD	Range
Cage trapped	92	0.29	0.27	0.15	0.04 - 0.85
Leg-hold trapped	5	0.12	0.05	0.16	0.01 - 0.41
Shot	7	0.23	0.21	0.08	0.10 - 0.33
Calorimetry 1 ^a	5	0.19	0.20	0.09	0.08 - 0.32
Calorimetry 2 ^b	4	0.39	0.36	0.19	0.23 - 0.64

a blood collected immediately after removal from calorimeter

b blood sample taken after 4 days recovery in indoor cages

A significant difference was found between at least one of the medians and the rest (corrected $\chi^2 = 10.5$, $p = 0.033$). The Kruskal-Wallis multiple-comparison Z-value test was applied to the results to determine which of the medians were significantly

different. Three pairs of medians were found to differ significantly ($p > 0.05$). The medians of these pairs and their respective z-values were: calorimetry 1 and 2 (2.00), leg-hold trapped and calorimetry 2 (2.59), and leg-hold trapped and cage trapped (2.45).

Simple linear regression of T_4 concentration (square root transformed, $n = 93$) derived from possum matrix calibrators and the concentration determined with canine calibrators (predictor variable), with the intercept forced through the origin, found the regression coefficient to be 0.736 (0.692 - 0.780), with an adjusted R^2 of 0.92.

The results of GLM analysis using repeated measures is presented in Table 10-XII. Increases in weight were found to be associated with higher levels of circulating T_4 . Seasonal effects were also significant and examination by the use of contrasts showed that significantly higher levels of T_4 were found in autumn than in any other season (Table 10-XIII).

Table 10-XII. Model examining the effect of weight and season on $T_4^{0.5}$ values in cage trapped possums. N = 161, includes results from 93 possums, some with repeated measures

Model variables	DF	F - value	p - value	Eta squared
intercept	1	57.9	<0.001	0.271
weight	1	19.9	<0.001	0.114
season	3	5.69	0.001	0.099

Adjusted $R^2 = 0.16$

Table 10-XIII. Estimated marginal mean serum $T_4^{0.5}$ concentrations for the four seasons, derived from the model presented in Table 10-XII. Those values with the same superscript symbol are significantly different from each other

Season	Mean $T_4^{0.5}$	SE
Autumn	0.614 ^{§††}	0.020
Winter	0.536 [§]	0.021
Spring	0.527 [†]	0.018
Summer	0.499 [†]	0.021

The most parsimonious model without repeated measures, examining the significant factors affecting T_4 levels (Table 10-XIV) was very similar to that found using repeated measurements. However, length and the interaction term of length and

weight were found to be significant. Increasing length being associated with a decline in T_4 , and increases of the interaction term of length and weight being associated with increased T_4 levels.

Table 10-XIV. Final model examining the effect of weight, length and season on $T_4^{0.5}$ values in cage trapped possums. (N = 93, includes no repeated measurements)

Model variables	DF	F - value	p - value	Eta squared
intercept	1	13.0	0.001	0.132
season	3	2.9	0.04	0.091
weight	1	1.0	0.317	0.012
length ³	1	5.0	0.028	0.055
length ³ *weight	1	3.9	0.050	0.044

Adjusted $R^2 = 0.11$

Although limited by the power of the contrast (results presented in Table 10-XV), autumn once again appeared to be the season associated with the highest T_4 levels.

Table 10-XV. Estimated marginal mean serum $T_4^{0.5}$ concentrations for the four seasons, derived from the model presented in Table 10-XIV. Those values with the same superscript symbol are significantly different from each other

Season	Mean $T_4^{0.5}$	SE
Autumn	0.577 ^s	0.027
Winter	0.538	0.029
Spring	0.492	0.026
Summer	0.471 ^s	0.030

Delta Con A

The models (both with and without repeated measures) examining the parameters found to have a significant influence on delta Con A are presented in Table 10-XVI and Table 10-XVII.

Table 10-XVI. Model examining the effect of site, age and weight on delta Con A. Based upon 745 measurements on 323 possums

Model variables	DF	F - value	p - value	Eta squared
intercept	1	1.52	0.217	0.002
site	3	7.53	<0.001	0.030
lnage	1	4.73	0.030	0.006
weight	1	11.98	0.001	0.016
lnage*weight	1	9.49	0.002	0.013

Adjusted $R^2 = 0.05$

In the model shown in Table 10-XVI, delta Con A was found to increase with greater possum age and weight, but to decrease with increases in the interaction term of possum age and weight.

Table 10-XVII. Model examining the effect of site, number of times captured, length and age on delta Con A. (N = 321, without repeated measurements)

Model variables	DF	F - value	p - value	Eta squared
intercept	1	25.03	<0.001	0.074
site	3	5.38	0.001	0.049
lnage	1	5.63	0.018	0.018
caught ^{0.25}	1	6.11	0.014	0.019
length ³	1	6.67	0.010	0.021

Adjusted R² = 0.07

In the above model (Table 10-XVII), delta Con A increased with increasing length and number of times caught, but decreased with increasing possum age.

The least squares means for the four sites (derived from the model shown in Table 10-XVI) are presented in Table 10-XVIII. Although the absolute values of the same means were different in the analysis involving no repeated measures, the ranking of the means was the same, suggesting that the analysis with repeated measures was valid. In both models the most influential parameter was the site of capture. The results suggest that there are significant differences in delta Con A responses between sites, and that possums dwelling around Queen St. may have depressed CMI responses, whereas those in the Southern section may have enhanced CMI responses, when compared to the balance of the population.

Table 10-XVIII. Least squares means of delta Con A from the four sections of the study site, derived from the model in Table 10-XVI. The same superscripts show the sites for which the means were significantly different

Site	Mean delta Con A	SE
Queen St.	398.3 ^{†‡}	15.5
North	434.8 [§]	9.7
Middle	445.5 ^{*†}	8.9
South	485.6 ^{*†§}	11.6

Analysis investigating the relationship between cortisol levels and delta Con A, utilising a subset of 259 possums where corresponding results were available for both parameters, failed to show any significant association between these variables.

Cholinesterase

One hundred and eighteen of the 130 samples provided useable results (for which the coefficient of variation was less than 20%). The data displayed a great deal of variability, with values distributed log-normally, necessitating a square root transformation to normalise the data. The untransformed data ranged from 227 to 8686 U/l with a median of 3621 U/l. The transformed data had a mean of 59.4 with a SD of 15.23.

The most parsimonious model arising from the GLM shown in Table 10-I, found that the only parameter with a significant effect on serum cholinesterase at the 5% level, was the sex of the possum ($p = 0.002$). The least squares means of serum cholinesterase^{0.5} for males being 62.8 ($n = 74$; $SE = 1.70$), and for females 53.7 ($n = 44$; $SE = 2.21$). The final model had an adjusted R^2 value of 0.08, suggesting that there was a great deal unexplained variation in the data.

Discussion

General comments

The use of repeated measures from a large number of individual possums has unbalanced the data and has introduced within animal, as well as between animal variation into the results. However, many individual possums were represented in the data set on multiple occasions, thereby ensuring that no single or small group of possums biased the results unduly. This is not thought to have invalidated the results of the study, as when analyses were conducted without inclusion of repeated measures, the results were essentially similar to those where repeated measures were used. This methodology, although failing strictly to comply with the requirement for independence of observations, was used to gain more power in analyses, and so improve the confidence in the resulting coefficients. The use of smaller data sets without repeated measures was also flawed. Because of the nature of the data, younger animals which were captured only once, are over-represented. This may have tended to lower the influence of site effects on the variable of interest, as these animals are more likely to have been transients, with little time spent in that section of the study site in which they were captured and bled.

These values for circulating cortisol and T_4 concentration, derived from the possum matrix calibrators were not used for analytical purposes in this study, as despite the systematic measurement error using the calibrators supplied, the results were still entirely satisfactory for the purpose of investigating the parameters which affect serum cortisol and T_4 levels in possums.

Climatic effects

Despite a number of approaches being taken, statistical models could not be found which showed that climatic variables had an impact on disease prevalence or incidence. However, this should not be taken to mean that climatic factors do not influence the disease process, but rather that the analyses were limited by low power, the crudity of the dependent variables, and the complexities of the host-agent-environment interactions. Other workers have faced similar difficulties when examining the interaction of climate and disease (Webster, 1981). However, Martin and Schwabe (1975) were able to show significant associations between weather variables and dairy calf mortality in California.

Pfeiffer (1994) found that the incidence of tuberculosis was significantly influenced by average mature male body weight, lagged 1 month. The most important meteorological factors which affected the cumulative incidence were shown to be the ratio of the monthly mean minimum to mean maximum temperature ($p = 0.0002$) and total monthly rainfall, lagged by 1 month ($p = 0.057$). Although these results were limited by the number of observations they were suggestive that weather and nutritional factors played a role in the development of clinical disease in possums. Examination of the charts shown in Figures 10-2, 10-3 and 10-4 suggests that the incidence of disease is responsive to prior weather conditions including lengthy dry periods and cool variable weather occurring 1 to 2 months beforehand. Deaths caused by tuberculosis appear to peak in both mid winter and summer, also suggesting that deaths in clinical cases are also hastened by factors associated with these two seasons.

Body weight and nutrition

Body weights at the study site are lower than many other populations, even those nearby, which suggests that they are living in an impoverished environment at high density (Fraser, 1979), where they are forced to eat less nutritious foods than they would prefer, and may as a consequence be more susceptible to stressful environmental effects. However, the population has good reproductive performance (Jackson, 1995), and with low death rates in juveniles compared with some other environments such as the Orongorongo Valley (Bell, 1981). This suggests that the genetic composition of the population may not allow the expression of a larger body size.

The body weight of possums clearly follows an annual cycle (Figure 10-7) in which the weight peaks in February after a period of feeding on nutritious spring/early summer growth. The decline in weight in the autumn presumably results from the lower energy and protein content of the available forage, as the decline in weight appears to precede the onset of the cooler wet weather of late autumn, which would increase energy requirements. However, a reduction in condition indices does not always coincide with a decrease in available nutrients (Humphreys *et al.*, 1984). Loss of body weight during the cooler months may not reflect low availability of nutritious forage, as both snowshoe hares (*Lepus americanus*) and bush rats (*Rattus fuscipes*) have both been shown to lose weight in the winter despite the provision of adequate food (Stewart and Barnett, 1983; Boonstra and Singleton, 1993).

Seasonal body weight fluctuations of possums in other environments are similar for males but different for females (Gilmore, 1969; Bamford, 1970; Jolly, 1976; Bell, 1981). In these studies female weights rose in autumn and peaked in the spring. This is may be due to the ripening of various fruits, both introduced and native, in the autumn and winter which provide a nutritious food source for the females (Jolly, 1976). The males in these areas may be more preoccupied with mating/agonistic activities during the autumn to take full advantage of the available fruit. Fruiting plants are not a feature of the Castlepoint study site, and thus body weight fluctuations probably follow the seasonal changes in the energy/protein levels in the preferred parts of available forage. The variation in seasonal body weight fluctuation between different environments, suggests that the findings at Castlepoint should be applied with caution to environments with a dissimilar range of plant species and climate.

The transient weight increase centred on the month of August (Figure 10-7), which occurs in both sexes, may be attributable to increased gut fill on pine catkins which are seasonally available on and near the study site. These are known to be a preferred food item of Castlepoint possums, and readily consumed (Paterson, 1993). The transient nature of this weight increase suggests that the nutritional value of pine catkins is sufficient for maintenance requirements only. Feeding on pine catkins for a 4 to 6 week period may provide an opportunity for increased social contact between possums, as the pine trees on the northern side of the site are few and scattered and do not extend into the adjacent property. Many possums are often visible by spotlight in isolated mature pine trees during late winter. This focus of activity on a food source could potentially provide an ideal opportunity for transmission of tuberculosis (Paterson, 1993), at a time of moderate nutritional and climatic stress, and may be the reason for the maintenance of a cluster of tuberculous possums on the north-eastern side of the study site.

Jolly (1976) found that 100% of possums on his Banks Peninsula study site visited introduced fruit trees with ripe or ripening fruit in the autumn. The possums moved up to 1600 metres to reach the focal food sources, where they congregated, fought and chest rubbed the trees. Green and Coleman (1981) also found that some possums in deep forest will regularly travel up to 1200 m to feed at the forest-pasture margin. Both Jolly (1976) and Hickling (1991) also believed this seasonal

focus of intense feeding and social activity was likely to provide an ideal opportunity for transmission of infectious disease.

Possum body weights were low, but increasing throughout the first 2 years of the study, then steadily declined for the next 3 years and fell precipitously in the final year (Figure 10-6). This pattern suggests that the drought prior to commencing the study regulated both the body weight and the population density (Appendix II) of possums at that time. Following the first severe winter, mild conditions prevailed for the next 3 years, allowing the population to gradually increase, with the mean body weight consequently declining. This mean body weight decrease was likely to have been hastened in the last year by the dry period commencing around Visit 50 (Figure 10-2), and the cool and variable winter and spring of 1993. Both these periods of weight loss were associated with subsequent peaks in the incidence of tuberculosis. The first epidemic apparently taking some years to subside, and concluding in the 7 month period in 1993 when no new cases of tuberculosis were identified, the prevalence maintained by only a single known infected possum (Figure 10-4).

Poor nutritional conditions have previously been hypothesised to have regulated possum populations. A population crash in New Zealand possums to 50% of pre-decline levels, was described by Thomas *et al.*, (1993), in which the decline was attributed to inadequate food supply and starvation, indicated by low mean body weights, very low levels of fat reserves, few pouch young and a low proportion of the population in the 1 year-old cohort. After the sudden reduction in possum density there was a significant linear increase in possum abundance over the following 10 years. A similar population response was observed in a Tasmanian forest recovering from fire (Hocking, 1981), whereby the growth rates, condition indices and reproductive performance of possums increased during the first six years following the fire, and then subsequently declined in association with higher possum densities. Although relatively stable, population fluctuations of up to 50% have also been noted in the Orongorongo Valley possums (Thomas *et al.*, 1993). Taken together this data suggests that the control of possum density is regulated extrinsically by changes in both the quantity and quality of available food resources, rather than by intrinsic controls such as social or spacing factors.

In humans protein-energy malnutrition has been shown to depress CMI responses, including DTH, and lymphocyte mitogenesis, although the possibility exists that specific vitamin (especially D₃) and mineral deficiencies may also be implicated (Harland and Brown, 1965; Law *et al.*, 1973; Neumann *et al.*, 1975; Rook *et al.*, 1986; Denis, 1991). Protein-energy malnutrition in mice has been shown to mediate some of its detrimental effects via impairment of respiratory burst activity in resident primed macrophages, and reduced phagocytosis and killing of intracellular pathogens (Redmond *et al.*, 1991). Unbound cortisol levels in malnourished children and swine are higher than normal, in response to decreased circulating corticosteroid binding globulin (Samuel *et al.*, 1976). Low serum albumin levels may also contribute to increased concentrations of unbound active GC in individuals suffering protein malnutrition (Neumann *et al.*, 1975). This suggests that animals with protein deficits will probably suffer from elevated levels of metabolically active GC, which Hill *et al.*, (1995) have shown in mice, to be a significant component of the immunosuppression caused by malnutrition.

Protein deficiency in guinea pigs has also been shown to produce a reversible loss of T cell reactivity to tuberculin, to impair DTH responses to PPD, and to decrease the efficacy of BCG vaccination (McMurray *et al.*, 1989a). Protein malnutrition also impaired the ability to control the accumulation of viable mycobacteria within lesions, as well as the haematogenous dissemination of bacilli to other organs. Complete and rapid reversal of immune dysfunction in guinea pigs occurs upon restoration of normal nutrition (McMurray, 1994). Investigations into the causes of recurrent tuberculosis infection of Irish cattle herds also implicated poor nutrition, including minerals and trace elements, as a contributing factor (Griffin *et al.*, 1993).

Hedgecock (1955) found that the type of dietary fat, and level of protein affected the resistance of mice to tuberculosis. Both low (10%) and high (30 and 40%) dietary protein levels increased the susceptibility of mice to infection. Ratcliffe and Merrick (1957) also showed that the susceptibility of Syrian hamsters to aerosol infection with *M. tuberculosis* was inversely proportional to the level of dietary protein. It was concluded that an adequate level of dietary protein was necessary for the long term resistance to infection. However, one important observation in these trials was the development of chronic progressive glomerulosclerosis in 50% of the infected hamsters on the high protein (30%) diet. Glomerulosclerosis did not occur

in the non-infected animals, nor those on lower protein (17 or 6%) diets. Ratcliffe and Merrick (1957) noted that reactivation of quiescent tuberculosis occurred in hamsters affected by the kidney disease. Kidney disease in humans is also a risk factor associated with reactivation of latent tuberculosis (Rieder *et al.*, 1989), and an association between chronic infection with mycobacteria and the formation of renal amyloid deposits is well established (McAdams *et al.*, 1983).

Kidney disease, including renal tubular casts, hyaline nephrosis with proteinuria, focal interstitial nephritis, pyelonephritis and renal amyloidosis, is also commonly found in older possums (Cooke, 1993; pers. obs.) and is thought to be responsible for weight loss in affected individuals. It is conceivable that renal disease, apart from causing weight loss and moisture stress during dry periods, may be one of the causal factors aiding reactivation of latent disease in older possums. The presence of mycobacteria may also precipitate renal disease in some possums, as noted in the Syrian hamsters above, and as observed in humans and sheep (McAdams *et al.*, 1983; Rings *et al.*, 1988). One moribund possum with severe renal disease and moderate lesions of tuberculosis has been found in the Castlepoint area. This matter requires further investigation.

Taken together the experimental evidence suggests that various dietary factors including protein and energy levels, type of lipids, trace elements and vitamins, all play a complex and interacting role in maintaining a protective CMI response to mycobacteria. In possums there is also the possibility of interactions between the disease process, diet and other pathologic processes, such as kidney disease.

Cholinesterase

In human medicine, blood proteins with short half-life have been used as diagnostic indicators of malnutrition, especially in patients undergoing surgery. As these proteins are produced in the liver, lower than normal levels reflect reduced synthesis due to a limiting supply of substrates. These blood proteins which include prealbumin, transferrin, retinol binding protein and cholinesterase have all been found at lower than normal levels in humans with both short and long-term protein-energy malnutrition (Waterlow, 1950; Ingenbleek, 1972; Neumann *et al.*, 1975; Young and Hill, 1978; Gofferje *et al.*, 1979; Shetty *et al.*, 1979; Haider and Haider, 1984; Ollenschläger *et al.*, 1989).

Measurement of serum cholinesterase, which has the shortest half-life of all plasma proteins (Ollenschläger *et al.*, 1989), was selected as the parameter to measure in stored sera, as it was believed that this may have provided us with a measure of whether individual possums were suffering from both acute and chronic protein-energy malnutrition, and whether low cholinesterase levels were associated with decreases in body weight, LTA responses and increased cortisol levels. The availability of a commercially manufactured kit, and the lack of species specificity, were determining factors in the use of cholinesterase measurements. It was also hypothesised that short (as well as lengthy) periods of malnutrition may have been important stressors inducing the reactivation of quiescent infections.

The measurement of cholinesterase levels proved to be an unproductive exercise as individuals showed wide and inexplicable variations in their serum levels. However, as pointed out by Ollenschläger *et al.* (1989), cholinesterase assays may be a more usefully employed where repeated measurements on individuals are performed, as the between-individual variation is too great to facilitate interpretation of single values. With the wisdom of hindsight, blood urea nitrogen (BUN) and serum albumin measurements may have been more usefully employed to monitor the nutritional status of possums. High BUN in many species is associated with elevated dietary protein intake (Franzmann, 1972; Lording, 1986), and in Australian possums highest levels have been recorded in the spring, and lowest levels in autumn (Viggers and Lindenmayer, 1996), corresponding to the expected protein levels in available forage. Elevated serum albumin levels occur with dehydration, and low levels with longer term protein deficiency of at least several weeks duration (Philbrick and Hill, 1974). Serum albumin may however, only prove to be useful as an indicator of severe and prolonged malnutrition, as it has a long half-life, with a large total body albumin mass on which to draw. The liver also has the capacity of to maintain synthesis, even during substrate shortages, and the catabolism and distribution of albumin from extravascular compartments may be altered during protein shortages (Shetty *et al.*, 1979; Haider and Haider, 1984). Previous studies in possums have shown increases in total serum protein levels with age, and low total protein levels have been recorded in lactating yearling females with low body weights (Barnett *et al.*, 1979). Elevations in serum protein also occur during the summer months (Presidente and Correa, 1981), and may be associated with dehydration.

Reproductive parameters

Changes in weight or decreases in population density have previously been shown to have minimal, or no effect on reproductive parameters, such as time of breeding, proportion of immature females breeding, or likelihood of spring births (Kean, 1971; Green and Coleman 1984; Keber, 1988; Cowan, 1993b). This study has confirmed these observations, although the data from the first year of the study was limited by the small numbers examined and inaccuracies in ageing offspring associated with the time of first head measurements. Cowan (1993b) hypothesised that there may be genotype differences between populations which affect reproductive performance, and which consequently reduce the ability to detect variation in reproductive parameters to environmental variation (Cowan, 1993b). For whatever reason, the use of reproductive parameters to demonstrate the effects of environmental stress appears to be a rather blunt epidemiological tool to show the significance of such effects.

Survival analysis in this study failed to show any significant association between sex and age at death in tuberculous possums. Late lactation in females was also shown to reduce the GC stress responsiveness of females (Table 10-IX). This may be analogous to the situation in rats (Walker *et al.*, 1992), women (Altemus *et al.*, 1995) and nesting birds (Wingfield *et al.*, 1995), where stress hyporesponsiveness of females with young or eggs has been observed. This centrally mediated phenomenon has been hypothesised to enhance the likelihood of successfully rearing offspring, through reduction of inappropriate metabolic and behavioural responses. This lowered stress responsiveness may be particularly important to possums in late lactation, as the young are no longer in the pouch, and are more likely to be neglected or underfed by a distressed mother.

Pfeiffer *et al.*, (1995) believed that there may have been an association between lactation in females an increased risk of tuberculosis, based upon his findings in studies of possums in the Hauhungaroa Ranges, where breeding females appeared to have a higher prevalence of infection. However, Jackson *et al.*, (1995a) also found no association between the number of tuberculous lesions and sex or the presence of pouch young. The current study does not support an association between lactation and an increased risk of tuberculosis (nor does that of Jackson *et al.*, 1995a), and in fact suggests that advanced lactation may have a sparing effect on stress responses.

Taken together, these observations suggests that gender related differences in physiology and reproductive status have little influence on the disease process.

In possum populations in which females commonly breed twice a year, such as at Castlepoint (Jackson, 1995), there will be an increase in the number of opportunities for disease transmission through intimate sexual contact and aggressive encounters during the mating period. Higher reproductive rates will also increase the opportunity for pseudo-vertical transmission by infected mothers. These phenomena may explain the association between the rearing of offspring and the increased prevalence of tuberculosis observed by Pfeiffer *et al.*, (1995). Despite tuberculosis being endemic in possums only a few kilometres distant, the possums of the Orongorongo Valley do not seem to be able to maintain the disease in their population, which has only one mating period per year, (Brockie *et al.*, 1987). By contrast, Pfeiffer *et al.*, (1995) found in the Hauhungaroa Ranges that the two areas in which possums commonly bred in the spring, also showed the highest prevalence of tuberculosis. However, high reproductive performance is only one of a number of factors likely to drive disease transmission. The extremely high prevalence of infection found at Flagstaff Flat (Coleman *et al.*, 1994a), occurred in a population in which spring breeding has been noted in a minority of females only (R. Jackson pers. comm.), and is testimony to the importance of other factors which facilitate *M. bovis* transmission.

Cortisol

General linear models investigating the parameters influencing cortisol levels explained between 19 and 22% of the variation in the dependent variable. Individual possum variation in the synthesis, release, utilisation and degradation of the hormone, diurnal rhythm of secretion and stress responses due to circadian rhythms (Khin Aye Than and McDonald, 1973; Seggie and Brown, 1975; Allen and Bradshaw, 1980; Breazile, 1988), and other unidentified factors, may have been responsible for considerable “noise” in the data.

Although haemolysis was not found to affect the concentration of cortisol or thyroxine detectable by radioimmunoassay in dogs, cattle and horses (Reimers *et al.*, 1991), it was thought prudent to investigate whether haemolysis had any effect on these assays in possum sera, as extrapolation of results from one species to another is often unwise. Possum serum samples are commonly affected by

haemolysis, even in those specimens collected with care and apparently little trauma to the red blood cells. Visual separation of the samples into four haemolysis categories, and subsequent analysis showed no significant effect of haemolysis on either the measured values of cortisol or T_4 . The period of storage also apparently had no significant effect on either cortisol or T_4 concentration in the sera.

In this study, unstimulated early evening cortisol levels in shot possums were found to have a mean value of 0.69 $\mu\text{g}/\text{dl}$. Normal resting levels of plasma/serum cortisol for possums have previously been reported to lie between 2.0 and 2.7 $\mu\text{g}/\text{dl}$ (Vinson *et al.*, 1973; Vinson, 1974a), which may indicate that these animals were undergoing adrenocortical stimulation at the time of sampling. Allen and Bradshaw (1980) found average total GC levels of 0.61 ± 0.08 and 0.75 ± 0.78 $\mu\text{g}/\text{dl}$ in a small number of male and female possums respectively, and Khin Aye Than and McDonald (1973) also found unstimulated total cortisol values of 0.92 ± 0.48 (SD) and 1.01 ± 0.57 $\mu\text{g}/\text{dl}$ in males and females respectively, both of which were similar levels to those found in this study.

Although females have larger adrenal glands than males, secretion rates per 100 mg of tissue are similar (Weiss and McDonald, 1966), and may partly explain why both resting and stimulated cortisol levels are higher in females (Figure 10-9). Studies in rodents have found that females also show higher GC stress responses (Seeman *et al.*, 1995). Higher stress responses also occur in female humans, although this is less marked than in rodents and occurs only after menopause. This increased stress response in females is thought to be largely due to the effect of oestrogens (Viau and Meany, 1991), although non-gonadal factors also probably have a role (Spinedi *et al.*, 1992). The higher cortisol responses in female possums may not indicate that they are innately more susceptible to the development of tuberculosis once infected, as there is no evidence to suggest that the severity of disease is sex-linked (Jackson *et al.*, 1995a).

In the shot sample, some cortisol may have been released agonally if the animals were not killed instantly and there was a delay in collecting the blood sample, as cortisol levels begin to rise within 2 minutes of stressor application in most species (Broom and Johnson, 1993). In this treatment category, a few individuals showing moderately elevated levels of cortisol (up to 2.91 $\mu\text{g}/\text{dl}$) may have been individuals which were not killed instantly.

Possoms have been shown to have similar responses to dexamethasone suppression tests and ACTH stimulation tests, as eutherians (Weiss and McDonald, 1966). Under stimulation by exogenous ACTH levels have been shown to rise to between 6.0 and 7.0 $\mu\text{g}/\text{dl}$, with higher responses recorded in females (Khin Aye Than and McDonald, 1973; Vinson, 1974a). These peak adrenal cortisol responses are within the range seen in both leg-hold trapped and cage trapped possums, which typically showed levels around 3.4 $\mu\text{g}/\text{dl}$ (Table 10-V). These stress responses between the two treatments were not significantly different, despite the leg-hold trapping being a more traumatic procedure. This amply demonstrates the limitations of using circulating GC measurements to gauge the severity of a noxious stimulus. The range in response is very limited and reaches an effective maximum relatively rapidly, and thereby prevents their use to differentiate between the intensity of stressors (Kant *et al.*, 1983). The normal stress response of possums seems to plateau with cortisol levels five times higher than those found in resting (shot) individuals, which is similar to the magnitude of GC response seen in other mammals, such as snowshoe hares, foxes (*Vulpes vulpes*), mice and cattle (Blecha *et al.*, 1982; Kreeger *et al.*, 1990; Shaw and Tume, 1990; Boonstra and Singleton, 1993). Some possums, low stress responders, release little cortisol even in response to such severe stimuli as leg-hold trapping, with cortisol values as low as 0.65 $\mu\text{g}/\text{dl}$ being found.

The lower cortisol levels of the possums which had been in the calorimeter, but sedated with ketamine hydrochloride prior to cardiac puncture, suggests that ketamine in itself does not stimulate release of cortisol, and that the period of captivity had accustomed the possums to a caged existence and acceptance of handling. Cold stressful conditions in the calorimeter prior to release did not appear to induce elevated cortisol levels, which accords with published observations where thermal stress has been shown to produce an acute rise in circulating GC only, followed by a period of normal GC levels despite the continued presence of the stressor (Maickel *et al.*, 1967; Minton and Blecha, 1990; Elvinger *et al.*, 1992).

Khin Aye Than and McDonald (1974), following experimental infusions of cortisol and ACTH, concluded that the possum is highly sensitive to the metabolic effects of GC, such as increased weight loss, urinary nitrogen excretion and glycosuria. It is also likely that their CMI may be similarly sensitive to the deleterious effects of

elevated circulating GC. Khin Aye Than and McDonald (1975) investigated the GC binding capacity of possum serum. Their conclusions were, that in the range of cortisol levels naturally encountered ($<10.0 \mu\text{g/dl}$), that approximately 80% is bound to corticosteroid binding globulin, 10% weakly bound to albumin and 10% is in the free active form. At total circulating cortisol levels of around $2.0 \mu\text{g/dl}$ Presidente and Correa (1981) showed that free cortisol levels were close to $0.3 \mu\text{g/dl}$. However, in possums which were stressed by capture and poor treatment, total and unbound cortisol levels rose to 4.23 and $0.72 \mu\text{g/dl}$ respectively, in the male possums examined. These abused animals showed signs of dehydration, a stress leukogram, gastric ulceration, colonic intussusception and rectal prolapse. However, in possums there is no suggestion from published accounts, or from this study, that possums, like the males of some dasyurid species (small marsupials) suffer from extreme elevation of free GC and a failure of steroid feedback mechanisms around the time of mating (Bradley, 1990).

As was expected, the number of times a possum was captured, had a significant influence on the circulating cortisol level. With habituation to cage trapping there was an associated decline in cortisol level.

The seasonal cycle of cortisol response shows a high summer peak indicative of a stressful summer period. This may be associated with heat stress, dehydration or food quality deterioration. In studies of biochemical parameters of *Trichosurus caninus* and *T. vulpecula*, serum protein levels were found to be highest in summer, which suggests that the possums may be suffering from mild dehydration at this time (Barnett *et al.*, 1979; Viggers and Lindenmayer, 1996). Nutritional factors may play a role, but cortisol levels were observed to rise through the spring, when food quality is increasing, and subsequently fall in the autumn when food quality is likely to be improving. This suggests that energy and protein levels in the diet do not have a significant influence on the cyclical summer peak stress responses observed.

The effects of constant heat stress on sows and sheep has shown that LTA responses are reduced, and that this reduction in lymphocyte activity was associated with an unidentified serum factor(s), but not circulating GC levels (Niwano, 1990; Becker and Misfeldt 1995). Heat stress on calves has also been shown to reduce the DTH reaction to PPD by 42% (Kelly *et al.*, 1982). Exposure of sheep to high

temperatures has been shown to cause persistent elevations in circulating prolactin levels (Parrot *et al.*, 1996), and it is likely that this hormone has immunomodulatory effects which may be detrimental to CMI responses (Khansari *et al.*, 1990; Draca, 1995). One way of assessing whether heat stress may be involved in detrimental physiological effects on possums, may be to monitor the resting temperatures of trapped possums throughout the four seasons, to determine if summer temperatures elevate rectal temperatures beyond the normal range. Detection of increased abnormalities in spermatozoa of animals (Finzi *et al.*, 1995) could also be used as a guide to the presence of heat stress. Serum albumin and total protein will also rise in dehydrated animals and is potentially another indicator of putative summer stressors.

The rise in cortisol levels during winter may be associated with periods of wet, cold weather and more severe temperature fluctuations which are prevalent at this time and will often produce conditions below the lower critical temperature of possums (van den Oord *et al.*, 1995). Social stresses associated with group feeding on the pine catkins may also exacerbate this peak. Prolonged cold stress has been shown to reduce DTH responses to PPD in calves by 14% (Kelly *et al.*, 1982). Chronic cold stress (2 weeks) in rats has also been shown to cause a persistent activation of pituitary-adrenal function. Studies in pigs have also shown that fluctuation in temperatures may be more conducive to immunosuppression, and susceptibility to transmissible gastroenteritis, than constant low temperatures (Shimizu *et al.*, 1978). The peak death rates noted in the summer and winter (Figure 10-5) may also be causally associated with the elevated cortisol levels seen during these same periods.

The only likely explanation for the minor peak seen in females in April and October is stress associated with mating activity which reaches a climax during these months. Females are known to be mobbed by a number of males in the period leading up to mating (G. Ward & C. Sauter pers. comm.), and the continual repulsion of unwanted sexual advances prior to oestrus may be stressful. Females have been noted to dominate these agonistic sexual encounters (Jolly, 1976), which could explain why they show cortisol stress responses during the mating period, whereas the males are less aggressive and can rest from the conflict when desired and hence show no cortisol stress responses associated with the mating period. Increased oestrogen levels in females have been shown to reduce circulating cortisol

(Khin Aye Than and McDonald, 1976), and are thus unlikely to have contributed to the higher levels observed during the mating period.

Over the period of the longitudinal study, the highest cortisol responses were associated with the start of the study, at which time body weights were low and the prevalence of disease highest (Figure 10-8). Over the ensuing years the cortisol levels fluctuated seasonally, with peaks each summer, but with an overall downward trend up till the termination. Although there was a moderate rise in prevalence of tuberculosis, and a decline in body weight in the final year, this was, inexplicably, not reflected in increased cortisol levels at that time.

Those possums which were known to have died (or likely to have died if not killed) showed a significantly higher cortisol response to cage trapping than the other two groups (Table 10-X). This suggests that the confirmed tuberculous group may have been more susceptible to disease progression following infection, as the other category, with LTA evidence of infection only, had a similar cortisol response to those with no evidence of infection. Furthermore, serial cortisol measurements showed no evidence of any association between infection or advanced disease and the level of serum cortisol. This is at variance with the disease in humans where adrenocortical insufficiency is commonly seen in tuberculous patients, and is believed to be proportional to the severity and chronicity of disease process (Uete, 1962; Beisel and Rapoport, 1969; Ellis and Tayoub, 1986; Srivastava *et al.*, 1980; Sarma, 1990). Although not statistically significant, analysis of CVs suggested that possums with LTA evidence of infection may have been spared from progressive disease, by not only showing low cortisol stress responses, but also through having less variable responses over time. In a population where the extrinsic levels of stress are high, those individuals with a genetically predetermined high GC stress response will be most at risk from infectious diseases requiring a competent CMI response for their containment or elimination (Mason, 1991) (Mason 1991). Chronic stress can thus cause suppression of innate resistance or acquired CMI, producing an animal with a compromised ability to deal with mycobacterial infection.

Thyroxine

Buaboocha and Gemmell (1995) found that the level of plasma T₄ in mature possums was 0.56 µg/dl. This compares to mean values of 0.23 µg/dl found shot

possums in the current study. This discrepancy, if real, may have arisen through non-specific interference of binding with the RIA kit used, but this possibility was not investigated.

The T_4 levels in leg-hold trapped possums were lower than the other treatment groups. Thyroxine and triiodothyronine (T_3) levels have also been shown to fall in response to the stress of leg-hold trapping in foxes (Kreeger *et al.*, 1990). This decrease in thyroid activity occur because of the effect of trauma and acute stress on neuroendocrine systems. Endogenous opioids, adrenalin and GC have all been implicated in the stress-induced reduction of circulating T_3 and T_4 in a variety of mammals (Brown-Grant *et al.*, 1954; du Ruisseau *et al.*, 1978; Judd and Hedge, 1982; Moore *et al.*, 1993; Messer *et al.*, 1995).

Immediately upon removal from the prolonged cold conditions inside the calorimeters, possums appeared to show normal T_4 levels. However, four days following removal, the T_4 levels had risen significantly, presumably as an adjustment response to the change in environmental temperature. This suggests that the high metabolic rate required in the calorimeters may have been maintained by noradrenaline-mediated thermogenesis, as has been observed in eutherian mammals (Withers and Hulbert, 1988).

General linear models explained little (11-16%) of the variation in T_4 levels and generally appeared to be unhelpful in understanding the epidemiology of tuberculosis. Increasing length and weight were associated with higher T_4 levels, which is in accord with earlier reports by Buaboocha and Gemmell (1995), who found peak levels of 4.5 $\mu\text{g}/\text{dl}$ in pouch young at 120 days of age, and which subsequently declined to adult levels. These high concentrations found in pouch young were thought to be associated with a period of rapid growth, changes in external morphology and the development of endothermy.

Thyroid hormones exert a calorogenic effect in both placentals and marsupials (Withers and Hulbert, 1988) and increased secretion occurs during short periods of cold exposure (Sojka, 1993). Thyroxine levels do vary acutely depending upon changes in ambient temperatures, but return to normal levels despite the continuance of the conditions which may have precipitated the alteration in hormone release. This may provide an explanation for the higher levels of T_4

observed during the autumn, and the low levels observed in the summer in the possums of this study.

Physiologic levels of thyroid hormones are required for normal function of CMI. However, the exact immunomodulatory mechanisms, and the effect of raised or lowered levels of thyroid hormones are unclear (Keast and Taylor, 1982; Chatterjee and Chandel, 1983; Williamson *et al.*, 1990), but unlikely to be of much importance as long as levels are maintained within normal physiologic limits.

Although there is an apparent association between elevated circulating GC and a reduction in levels of T_3 and T_4 in a number of species (du Ruisseau *et al.*, 1978; Moore *et al.*, 1993), no such association was found in the possums of this study. Although it was hoped that T_4 levels may have been able to provide a useful guide to the climatic/thermal stresses acting on possums, both between months and sites this did not eventuate. The disappointing assay results, models of limited utility, and difficulty of interpretation of results, led to a discontinuance of plans for further T_4 assays on the sera.

Delta Con A

Neither of the models (Table 10-XVI and Table 10-XVII) provides a good explanation for the variation in delta Con A results, as indicated by the low R^2 values (0.05 and 0.07). This may have arisen because of the manipulation of the delta Con A necessitated by the between-test episode variation. However, another difficulty encountered when interpreting LTA results is the enormous differences which exist among normal individuals and the variation in the same subject at different times (Yu and Clements, 1976).

An increase in the number of times a possum had been captured brought about a concomitant rise in the delta Con A value. This suggests that habituation to the stress of capture reduced the intensity of the reaction which depressed the ability of lymphocytes to respond to plant mitogens. In studying the differences between haematological profiles and lymphocyte mitogenic responses between first caught possums and those habituated to periodic capture, Buddle *et al.*, (1992) found that single capture possums had significantly lower mean lymphocyte and eosinophil counts, and lymphocyte stimulation indices, and higher neutrophil counts, than possums caught on multiple occasions. Although design problems necessitated

caution in the interpretation of the results, there appeared to be true differences in the stress response between the groups. Although a decrease in cortisol stress responses also occurs with habituation to trapping, and other research has shown that exogenous GC administration will reduce T-cell mitogenesis induced by Con A (Blecha and Baker, 1986; Wallgren *et al.*, 1994), no relationship between the levels of cortisol and delta Con A could be established in this study.

Decreases in lymphocyte proliferation have been seen in response to the application of unpredictable stress in both human and rodent studies, in the absence of significant elevations in circulating GC (Mormede *et al.*, 1988; Zakowski, 1995). This suggests that at physiologic levels, GC have little influence over lymphocyte mitogenesis. In human subjects reduced Con A lymphocyte responses were associated with increased blood pressure (Zakowski, 1995), suggesting the involvement of the sympathetic nervous system. This is consistent with research showing that immune tissues are directly innervated by sympathetic neurones and that lymphocytes bear adrenergic receptors (Bourne *et al.*, 1974). The response is not likely to be caused by elevated circulating catecholamines, as Yu and Clements (1976) found that administration of adrenalin to humans did not alter lymphocyte mitogenic responses to PHA.

Site effects

The examination to trap ratio provided a proxy for the density of possums living on or close to the four sections of the study site. Queen St. clearly had the highest yield of possums which suggests that this was a favoured site for possum denning, but this high yield may have been influenced by boundary effects, whereby possums living in the adjacent non-trapped area were frequently captured, as this section had the largest peripheral boundary length per area trapped, when compared with the other three sections.

The weights of possums were significantly higher on the Middle section and Queen St, than on the other areas of the study site (Table 10-III). This suggests that the possums from Queen St. and the Middle had more nutritious food available or were better sheltered than those from the other parts of the study site. Field observation would suggest that a greater variety of herbage was available to possums from the sites with the heavier possums, and that the gorse growing in those areas may also

provide better shelter. Barnett *et al.*, (1979), in a study of *T. vulpecula* in Australia also identified significant microhabitat-related differences in body weight.

Effects of microhabitat on cortisol levels were evident in the data. Circulating cortisol levels were found to be lowest for Middle section possums, and these levels were significantly different from the Northern and Southern sections, thus suggesting that that the Middle site (and possibly Queen St.) were intrinsically less stressful microhabitats for possums than other parts of the study site. This appeared to be associated with better nutritional or shelter conditions, as possums from these sites also had the highest body weights, despite the trap yield (possum density) being as high, or higher, in these sections than elsewhere on the study site.

The persistence of tuberculosis in these apparently more favourable sections of the study site suggests that the disease presence is associated with environmental factors other than those which might induce stress in the possums e.g. foci of social activity or sharing of den sites. Such persistent clusters of infection are a well recognised feature of the disease in possums (Hickling, 1991; Morris and Pfeiffer, 1995), and there is evidence that these are associated with local aggregations of possums, where animals are 4-16 times as crowded as if they were distributed randomly in the environment (Hickling, 1995).

In an attempt to establish whether unfavourable environmental variables affected the establishment of spatial clusters, Caley (1996) examined and compared the characteristics of possum den sites from areas where tuberculous possums had, or had not been captured over a number of years, in a farmland area near Taumarunui. The results failed to demonstrate any significant difference in the measured quality of den sites used by possums. The conclusion of the study was that if den quality does play a role in the origin of tuberculous clusters of possums, then that quality difference is difficult to detect, especially with tests of low-to-moderate power. The occurrence of possum crowding at 'hot spots' suggests that environmental conditions favourable to possums exist in these areas, rather than the reverse, as suggested by Morris and Pfeiffer (1995).

The section of the study site in which possums were captured, was one of the few variables shown to have a significant effect on the delta Con A responses. Possums in Queen St. had the lowest mitogenic responses, whereas those in the Southern

section had the highest responses. This suggests that possums living in Queen St. may have had their CMI responses compromised by environmental conditions. These adverse conditions may have included social stresses associated with an apparently higher population in the Queen St. area, or could be related to the more exposed aspect on top of a ridge with low vegetation, and which was often subject to strong winds, and the heat of summer. However, these delta Con A findings seem to contradict the more reliable findings of both the body weight and cortisol analyses, whereby these sections of the site appeared to be the least stressful. For the present, this apparent contradiction may have to remain an inexplicable paradox.

Conclusion

The influence of stress on immunity is mediated not only by glucocorticoids (GC), but also by catecholamines, endogenous opioids and pituitary hormones, such as adrenocorticotrophin (ACTH), arginine vasopressin (AVP), prolactin and growth hormone (Griffin, 1989; Lysle and Coussons-Read, 1995), so that in any compartment of the immune system multiple immunomodulatory mechanisms will be operating simultaneously. Blood samples analysed for cortisol fail to give any indication of the involvement of other hormones or the nervous system in the stress response of an animal, and the effect that these may have on cell-mediated immunity. Thus, although circulating cortisol proved to be a useful parameter to measure, it is still only one of a suite of factors, some known and others unknown, which could be used to gain insight into the physiology of stress and the effects on tuberculosis.

There are probably critical threshold levels to an infectious agent with which a host can peacefully coexist. Subtle, but functionally incapacitating effects of stress may disrupt the threshold and upset the balance between the agent and the host, thus precipitating disease. An experimental model of such a disease-host-environment relationship has been described by Porter *et al.*, (1984). In this model combined administration of an immunosuppressant (cyclophosphamide) and Venezuelan equine encephalitis virus to pregnant female white mice and wild deer mice, in combination with moderate limitation of food and water, resulted in reduced growth or reduced survival of young to weaning. However, when food and water were adequate the two species compensated and brought young to weaning, albeit at weights below control values. Results suggested that when environmental

conditions are satisfactory that disease and other factors producing morbidity can be well controlled, but compensatory mechanisms fail once water deprivation and protein-energy malnutrition provide additional stress. This may be analogous to the situation in possums where there is accumulating evidence to suggest that many possums can be exposed to *M. bovis*, and subsequently remain as healthy “sleepers” until challenged by a combination of stressors (Chapter 9).

Disease progression in possums may be due to uncontrolled replication of mycobacteria in macrophages. This may be permitted in Bcg^s genotypes, or equivalent (Frelier *et al.*, 1990; Zwilling *et al.*, 1990), or by phenotype alteration induced by stressors affecting macrophage competence, and lymphocyte responses, resulting in impaired intracellular killing of *M. bovis* and poor antigen presentation. This may commence a T-helper 2 cascade of inappropriate immune responses (Mason, 1991; Ferrick *et al.*, 1995; Pollock *et al.*, 1996).

There is a developing picture of major environmental stressors precipitating outbreaks of endogenous reactivation disease in “sleepers”. These index cases are highly infectious, distributed widely in the environment, and are likely to initiate a widespread disease epidemic in a pre-stressed susceptible population. This epidemic may take several years to subside and during this period of falling prevalence, the number of clinical and infectious individuals gradually declines and the disease retreats, persisting in clinical form only at sites where environmental conditions favour transmission and/or progression of the disease. Variation in the individual response to infection overlays this scenario, with individuals with low disease resistance (including high stress responders) being principally responsible for the continued presence of clinical cases and deaths in the population, between the catastrophic climatic events which trigger epidemics.

Pfeiffer (1995) found no correlation between possum density indices and prevalence of tuberculosis in the Hauhungaroa Ranges. However, Coleman (1988) found the highest prevalence of tuberculous possums (33.3%) in the Hohonu Range was in areas with rough pastoral grazing, where there was a low density of possums, which were readily trapped to extinction. Later observations made by Coleman *et al.*, (1994a) also found the highest prevalence of tuberculosis in possums associated with sub-maximal densities at Flagstaff Flat on the West Coast. The highest prevalence of disease (20%) at the Castlepoint study site was also found to be

associated with the lowest population density (Jackson, 1995). The apparent absence of human influence on the populations cited, suggests that either tuberculosis or some other adverse environmental factor, or both, was responsible for the sub-maximal possum densities in the areas concerned, or that tuberculosis prevalence is entirely density independent.

If the prevalence of tuberculosis is density independent, then transmission mechanisms are likely to revolve around opportunities in which aggregated social activity occurs, and opportunities for intimate contact are common. Given that concurrent and even sequential den sharing is uncommon at Castlepoint (C. Sauter pers. comm.), such opportunities are likely to occur on three occasions per annum on the study site. Two such opportunities are associated with the mating periods in April and October where groups of males follow individual females in the hope of successful copulation (Jolly, 1976; G. Ward pers. comm.). During these events there are multiple agonistic encounters, often involving hissing, biting and scratching, between males, but particularly between males and females. Another opportunity is likely to arise when possums aggregate at foci of mature radiata pine trees during the period from July to September to feed on the pollen containing catkins (Paterson, 1993). Possums through this period have long been recognised to feed on pine catkins, and their faeces takes on a resultant yellow hue. Jolly (1976) also found these feed-associated foci of possum activity to be accompanied by intersexual agonistic behaviour. The multiple opportunities for transmission throughout the year, and the potential for development of latent infections and endogenous disease reactivation with stress, would make identification of a pattern of tuberculosis acquisition difficult to identify. Nixon (1989), in a study of the seasonal occurrence of intraspecific wounding in possums found considerable temporal variation in its occurrence between areas, with peaks often corresponding to times of mating activity, but also with high levels in January, February and March in some areas, further suggesting that aggregation and fighting around seasonal food sources may be widespread.

The findings of this study suggest that glucocorticoid assays and monitoring of trends in body weight, are useful tools for investigating stressful environmental phenomena, and helpful in uncovering aspects of the epidemiology of tuberculosis in possums. Major stressful periods involving inadequate nutrition, heat, cold and

moisture stress appear to precipitate severe tuberculosis outbreaks, which are believed have their origins in the reactivation of subclinical/latent infection in the susceptible portion of the population (Chapter 9). As the period of pre-clinical disease varies substantially, and can be as long as several years, this epidemic of tuberculosis takes several years to subside. Thereafter a small number of clinically diseased possums are likely to be restricted to “hot spots” conducive to transmission of infection.

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The PhD candidate was responsible for managing the Castlepoint study site from July 1993 till April 1996. Hormone and cholinesterase assays were conducted by the candidate. Data collection and storage (with assistance) through this period, and subsequent analyses have been carried out by the candidate.

CHAPTER 11

**RESTRICTION ENDONUCLEASE ANALYSIS
(REA) RELATIONSHIPS AMONGST *M. bovis*
ISOLATES FROM AROUND CASTLEPOINT**

Abstract

Isolates of *M. bovis* recovered from a variety of species, both wild and domestic, in the Castlepoint environs, and in particular the Castlepoint study site, were subjected to restriction endonuclease analysis (REA) to DNA fingerprint the strains present in this locality, and hence gain a better understanding of the inter- and intraspecific epidemiology of tuberculosis. In this area, which covered approximately 240 km², isolates from 284 animals were REA typed. From the nine mammal species examined, which included cattle, deer, pigs, goats, sheep, possums, ferrets, cats and hedgehogs, there were 20 distinct REA types identified, with eight of these occurring on the 21 ha study site.

Data arising from this study of *M. bovis* strains has not challenged the view that possums are major reservoir hosts of tuberculosis in the Wairarapa. There was also no evidence to suggest that host adaptation of *M. bovis* has occurred, except in the case of possums, where they appear to be able to maintain clusters of individuals infected with particular restriction types, in microhabitats for at least 5 year periods. The occurrence of newly introduced restriction types into these endemic clusters has made possible new observations on the epidemiology of infection, including the documentation of the occurrence of latent infections, duration of primary progressive disease in newly infected possums (7-8 months), and the likely occurrence of post-primary reactivation of tuberculosis.

Introduction

Traditionally, understanding of the epidemiology of bovine tuberculosis has been hampered by the lack of accurate and sensitive methods to differentiate between isolates of *Mycobacterium bovis*, the causative organism. Conventional methodology involving the identification of phenotypic markers such as biochemical reactions, specific antigens, phage typing and antibiotic sensitivity have not been successfully exploited for mycobacteria, mainly owing to the remarkable uniformity within the species (Grange *et al.*, 1990; Butcher *et al.*, 1996). The development of highly discriminative DNA based techniques has recently led to an improved ability to trace the source of *M. bovis* infections in man, domestic stock and wildlife (Collins *et al.*, 1986; Collins *et al.*, 1988; de Lisle *et al.*, 1990; Collins *et al.*, 1994; van Soolingen *et al.*, 1994; Bolske *et al.*, 1995; Cousins and Williams, 1995; de Lisle *et al.*, 1995; Gutiérrez *et al.*, 1995; Perumaalla *et al.*, 1996; Romano *et al.*, 1996; Liebana *et al.*, 1997), and develop to develop sound hypotheses regarding the epidemiology of infection.

Restriction endonuclease analysis (REA) is an electrophoretic separation of DNA fragments following digestion of chromosomal DNA, which can reveal subtle differences between strains on the basis of a mobility shift in one or more fragments, or the loss or acquisition of fragments. REA types have been shown to be stable and provide a valuable tool for studying patterns of *M. bovis* transmission (Collins *et al.*, 1986; Collins *et al.*, 1988; de Lisle *et al.*, 1990; de Lisle *et al.*, 1995). However, a very large number of fragments are produced which makes the electrophoretic conditions critical for good results, and the subsequent analysis of gel banding difficult. REA has been used to effectively differentiate strains of *M. bovis* which show little variation with IS6110 (Collins *et al.*, 1993).

Because of the difficulties associated with achieving reliable results with REA analysis, alternative simpler techniques are commonly used by many laboratories to type *M. bovis* strains. These include restriction fragment length polymorphism (RFLP) and polymerase chain reaction (PCR) based methods. RFLP involves the hybridisation of DNA fragments with specific probes. The number of fragments is much reduced when compared with REA analysis, thus simplifying the identification of broad differences between isolates, at the expense of missing substantial genotypic variation. Insertion sequences, and other repetitive elements

(DR) are often used as probes, due to variation in copy number and genomic location. The insertion element IS6110, typically present as multiple copies in *M. tuberculosis*, appears as a single copy in the majority of *M. bovis* strains, including those in New Zealand (Collins *et al.*, 1993; van Soolingen *et al.*, 1994; Romano *et al.*, 1996), which severely limits the power of this technique for strain differentiation in *M. bovis*. However, *M. bovis* strains harbouring multiple copies of this element have been described in wild animals, goats, and humans and RFLP with IS6110 has been successfully used to characterise outbreaks of tuberculosis caused by *M. bovis* (van Soolingen *et al.*, 1994; Liébana *et al.*, 1997). Recently it has been shown, that by combining patterns generated by DR and polymorphic GC-rich repetitive sequence (PGRS) of digested DNA, that it is possible to significantly increase the strain differentiation possible using RFLP (van Soolingen *et al.*, 1994; Gutiérrez *et al.*, 1995; Perumaalla *et al.*, 1996; Romano *et al.*, 1996).

Polymerase chain reaction methods for amplification of DNA, are currently being developed for typing mycobacteria, as potentially they do not require the extraction of large amounts of DNA. This may, in the future, allow typing to be done directly from clinical specimens. However, this technology is still in developmental stages and has only recently been subjected to trials to assess the ability of the technique to differentiate *M. bovis* isolates (Glennon *et al.*, 1997).

DNA fingerprinting using REA has been used extensively in New Zealand to identify the source, and the relationship between *M. bovis* isolates from outbreaks of tuberculosis in domestic stock and wildlife in the same area (Collins *et al.*, 1988; de Lisle *et al.*, 1990; Atkinson and Cowan, 1994; de Lisle *et al.*, 1995; Ragg *et al.*, 1995a). Overall the results have shown that the REA types identified in wildlife and domestic stock in the same area have been the same or very closely related. There have been no reports of ferret and domestic stock isolates of *M. bovis*, from the same locality, having distinctly different REA types (D. Collins pers. comm.).

This chapter describes the investigation of bovine tuberculosis strains in the Castlepoint area, in the Wairarapa, a region with at least a 40 year history of endemic *M. bovis* infection in wildlife (Ekdahl *et al.*, 1970). The investigation, as well as relying on data arising from the longitudinal study of tuberculosis in an infected possum population (Pfeiffer, 1994; Jackson, 1995), also made use of data arising from the slaughter of bovine skin test reactors, cross sectional studies of

infected possum populations and opportunistic sampling of wild mammals. The characterisation of the REA types of *M. bovis* isolates from multiple species within about 12km of the longitudinal study site formed the basis of the study.

The purpose of this investigation was to clarify whether there were any discernible patterns in the distribution of REA types between host species in the Castlepoint environs, and more specifically, to investigate whether there was any substantial REA evidence to suggest that any particular species, other than possums, may have been a reservoir host of *M. bovis*. The temporal distribution of incidence of REA types on the study site was also investigated to gain insight into the epidemiology of tuberculosis in possums. Detail on the spatial relationships of infected possums and their REA types is the topic of another investigation being conducted by J. McKenzie.

Materials and Methods

The data reported in this chapter has principally arisen from the Castlepoint longitudinal study, briefly described in Chapters 9 and 10. More comprehensive descriptions are provided by Pfeiffer, (1994) and Jackson, (1995).

Study area

As well as focusing on the isolates obtained from animals on the longitudinal study site and immediate surrounds, the examination of isolates from further afield was undertaken. This area, termed the “greater Castlepoint” environs encompassed an area approximately 22 km long by 11 km deep. This was centred upon the study site on Waio station and was bounded to the east by the Pacific Ocean (Figure 11-1).

Acquisition of isolates

In the course of this study all isolates of *M. bovis* from wildlife around the study site and Castlepoint area were REA typed at the AgResearch Tuberculosis Laboratory, Wallaceville, Upper Hutt. During the longitudinal study, skin test negative steers were run on the study site and skin tested at approximately 3 monthly intervals. Reactors were removed, slaughtered and necropsied. Lesions found were submitted for culture and REA typing. Twelve domestic deer were also depastured on the study site during 1993/94 (Chapter 5), and of these five became infected with *M. bovis* and the infecting REA type determined.

In addition to the restriction typing conducted on isolates from around the study site, local farmers were requested to make any suspect wild animals from the general area available for necropsy and culture where possible. In addition, a variety of hunting forays were organised by the manager of Waio station or a close neighbour. These expeditions provided numerous possums, pigs and deer for necropsy. The neighbour to the north was also encouraged to submit specimens of wildlife that he or his sons shot or trapped on their property. This resulted in a number of deer, goats, hedgehogs, possums, rats and rabbits for necropsy. Requests for reactor cattle isolates from the Castlepoint area to be REA typed were also made of the Tuberculosis laboratory, which routinely handled such specimens. Data which already existed on REA types from cattle and other species in the area were also made available by the AgResearch Tuberculosis laboratory and the Ministry of Agriculture and Fisheries office in Masterton.

From the greater Castlepoint environs, the oldest REA data was from possum isolates gathered in 1982. The data from the longitudinal study covered the period from its start in April 1989, up till and including the termination in October 1994.

DNA restriction endonuclease analyses of *M. bovis* isolates was carried out using the method described by Collins and de Lisle (1985). The procedure was modified slightly, such that three, and not two restriction enzymes, were used to further aid strain discrimination in the analyses (Collins *et al.*, 1986). Strains were characterised and compared on the basis of their DNA fragment patterns on lengthy agarose gels.

Restriction types have been reclassified to simplify the understanding of the disease epidemiology in the Castlepoint area. The previous alphanumeric system introduced in Chapter 5, and used in the theses of Jackson (1995) and Pfeiffer (1994), had been originally adapted from the numeric classification of isolates reported by Collins *et al.* (1986). This old system was discarded in favour of a simple alphabetic system. Restriction types from the study site took the letters from A to H, with the most numerous isolate denoted as REA type "A" and the least numerous isolates given later letters of the alphabet, finishing with the letter "T" for isolates from the greater Castlepoint area (Table 11-I). This change was introduced as it was feared that REA types with the same numeric prefix, may have been incorrectly presumed to have been more closely related to each other than those

with a different prefix. Although there is genetic variability amongst the REA types, often shown by only one or two bands difference on the gels, D. Collins (pers. comm.) was reluctant to develop dendrograms showing the apparent genetic divergence and relationships between types, as he felt the task too complex and the results likely to be unreliable.

With the re-examination of some of the earlier study site gels, there has also been a minor reclassification of REA types from the study site which has produced results slightly at variance to those reported in the theses of Jackson (1995) and Pfeiffer (1994). The major changes to REA classifications previously reported are that types 4, 4a, 4b+c and 10 are now called A, B, E and D respectively.

Results

Greater Castlepoint environs

From the nine species from which *M. bovis* were recovered there were 284 isolates which were REA typed. Twenty distinct REA types were identified in the greater Castlepoint area which covered approximately 240 km² (Table 11-I). No individual was found to be infected with more than one REA type of *M. bovis*.

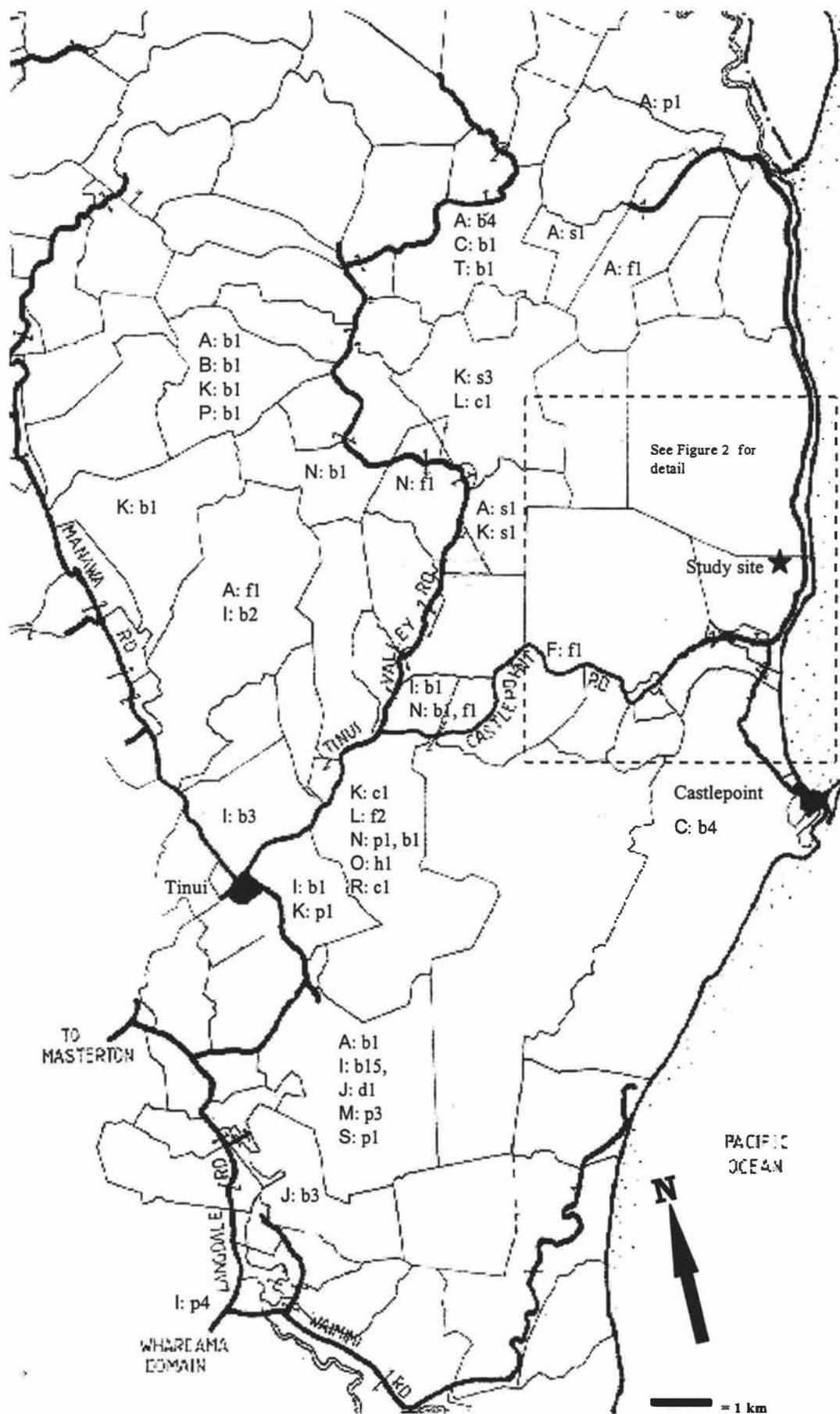
Particularly widespread of the restriction types were those previously described by Collins *et al.* (1986) i.e. types A (was type 4) and I (was type 8). Type A was found over the whole of the area shown in Figure 11-1, whereas type I was more prevalent in the south and west of the area shown. Type A is known to have a very wide distribution also being found as far north as Dannevirke, Akitio and Pongaroa and as far west as Featherston (D. Collins pers. comm.). Type I, however, has a more southerly distribution occurring several kilometres to the west of Tinui and as far south as Homewood. Apart from the two restriction types mentioned above, the other strains identified appear to have smaller geographic distributions, although interpretation is limited by the low number of isolates available for most of the strains.

Table 11-I. Restriction type and number of animals infected in the greater Castlepoint area (data arising since 1982)

REA type	Total	Possum	Cattle	Swine	Deer	Ferret	Cat	Goat	Sheep	Hedgehog
A	96	45	18	12	8	7	1	2	1	2
B	56	41	12	1	-	-	-	1	1	-
C	35	10	14	3	5	3	-	-	-	-
D	17	8	4	1	-	3	-	1	-	-
E	19	12	4	2	1	-	-	-	-	-
F	3	2	-	-	-	1	-	-	-	-
G	1	1	-	-	-	-	-	-	-	-
H	1	1	-	-	-	-	-	-	-	-
I	26	4	22	-	-	-	-	-	-	-
J	4	-	3	-	1	-	-	-	-	-
K	8	1	2	4	-	-	1	-	-	-
L	3	-	-	-	-	2	1	-	-	-
M	3	3	-	-	-	-	-	-	-	-
N	6	1	3	-	-	2	-	-	-	-
O	1	-	-	-	-	-	-	-	-	1
P	1	-	1	-	-	-	-	-	-	-
Q	1	-	-	1	-	-	-	-	-	-
R	1	-	-	-	-	-	1	-	-	-
S	1	1	-	-	-	-	-	-	-	-
T	1	-	1	-	-	-	-	-	-	-

Restriction type B was found principally on the study site, but isolates have also come from a goat and a possum from the adjacent property to the north, and also from a bovine approximately 9 km to the north-west. This suggests that although this type is the second most common on the study site, that overall numbers of animals infected and the distribution is likely to be quite restricted. Type C (Figure 11-2), although moderately common on the study site, appears to be confined to properties immediately adjacent to the study site. Details of the occurrence of other REA types are available from Table 11-II and Figure 11-1.

Figure 11-1. (Over page). Map of Castlepoint and environs showing roads and property boundaries. REA type, species and number of isolates are overlaid on properties or area of origin. Upper case letter = REA type, lower case letter indicates species infected i.e. b = bovine, c = cat, d = deer, f = ferret, g = goat, h = hedgehog, o = ovine, p = possum, s = swine



Longitudinal study site and immediate environs

On the study site, which has an area of approximately 21 ha, eight REA types of *M. bovis* were identified. Of 109 possums believed to be tuberculous (not including those ELISA or LTA positive), REA typing was successfully performed on isolates of *M. bovis* from 102. Of the possums on the study site for which REA typing data was available, there were 39 with type A, 35 with type B, seven with type C, two with type D, eleven with type E, and one each with types F, G, and H (Table 11-II). The restriction types of *M. bovis* isolates obtained from possums and other mammals on and around the study site are shown in temporal sequence in Figures 3 and 4 respectively. The 21 isolates presented in Figure 11-4 came from both domestic deer (5) and cattle (9), as well as wild or feral mammals, which included two each of ferrets, pigs, and sheep and one hedgehog.

From the numbers of possums infected with each strain, it is apparent that types A, B and E are endemic on the site or immediately adjacent areas, whereas type C may possibly be endemic on the site. Types D, F, G and H are likely to have been transient strains, introduced by immigrants from areas nearby in which these types are well established or alternatively, especially in the case of types G and H, their presence may be evidence of mutations causing the development of new REA types which failed to persist, as these strains were not isolated elsewhere.

Table 11-II. Restriction type and number of animals infected in the study site and immediate area (April 1989 - October 1994)

REA type	Total	Possum	Cattle	Swine	Deer	Ferret	Sheep	Hedgehog
A	50	40	3	2	3	-	1	1
B	41	37	3	-	-	-	1	-
C	13	7	3	-	2	1	-	-
D	4	3	-	-	-	1	-	-
E	12	12	-	-	-	-	-	-
F	1	1	-	-	-	-	-	-
G	1	1	-	-	-	-	-	-
H	1	1	-	-	-	-	-	-

One of the most prevalent REA types on the study site in the first 2 years was type A. However, this strain was never as common, and failed to persist as well as type B in the following 2 years. The final year of the study saw a resurgence of strain A, such that it became the most prevalent REA type found in possums during the termination of the first phase of the study, with most infected possums coming from

the country bordering the study site to the north and west. This suggests that this strain had persisted in a cluster of possums adjacent to the study site, perhaps for the 5.5 year duration of the longitudinal study.

REA type B was prevalent at the commencement of the longitudinal study in April 1989, but the number of cases dwindled over time such that no new cases were identified in the 21 months prior to the termination in September/October 1994. During the termination three new cases of this strain were identified in previously untrapped possums. These three cases were found next to the windbreak row of large pine trees adjacent to the northern boundary of the study site. This suggests that the existence of this REA type on the site may have been in jeopardy, as there was only one long-surviving, non-infectious individual which was known to carry this strain on the study site. Furthermore, this provided evidence that a strain of *M. bovis* can go into a significant "population decline", presumably due to chance events which do not favour bacterial transmission.

REA type C was initially isolated from a ferret captured on the site in September, 1990. Later it was again found in a single possum in the third year of the study (Figure 11-3). This animal was a 4 year-old female (Case 2418), which had been present and regularly captured since the start of the study. She died from tuberculosis in September 1991, and her 5 month old offspring also perished at the same time. She had no spring-born offspring, so was unlikely to have been sexually active during the period in which she may have been infectious. However, the first of the next group of possums (Case 2841) which were found to have REA type C was first captured on the site in January 1992. This male was a 2.5 years old at first capture, and subsequently showed LTA evidence of infection in March 1993 at its first test. A latter two LTA tests were both negative. This possum died from tuberculosis in January 1994, and was the first of a group of five previously captured possums (three males and two females) which all succumbed to infection from this strain, and died in April and May 1994. One previously untrapped possum was also found with advanced infection with this strain at the termination in October 1994.

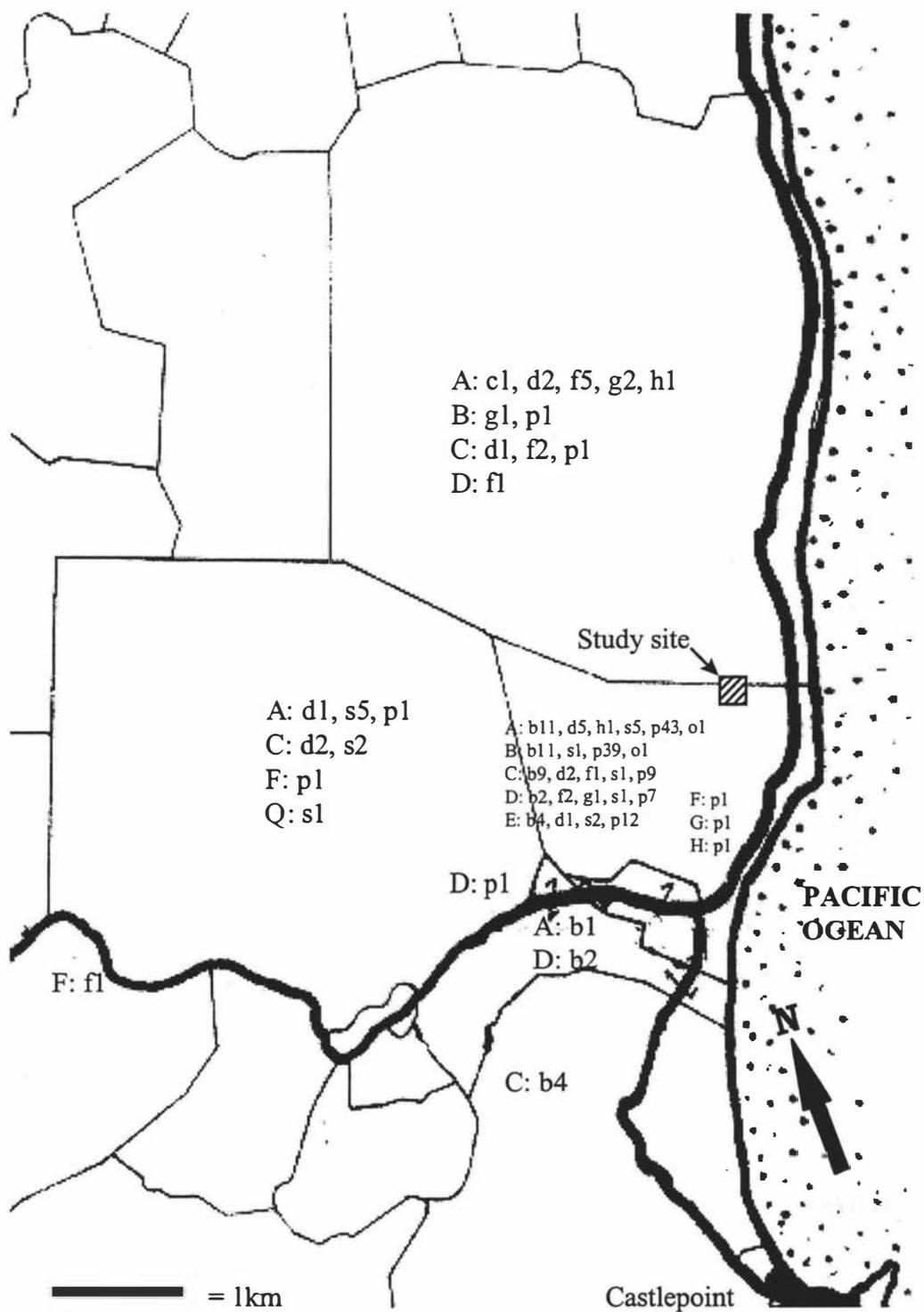


Figure 11-2. Detail of the REA type, species and number of isolates from the map insert shown in Figure 11-1.

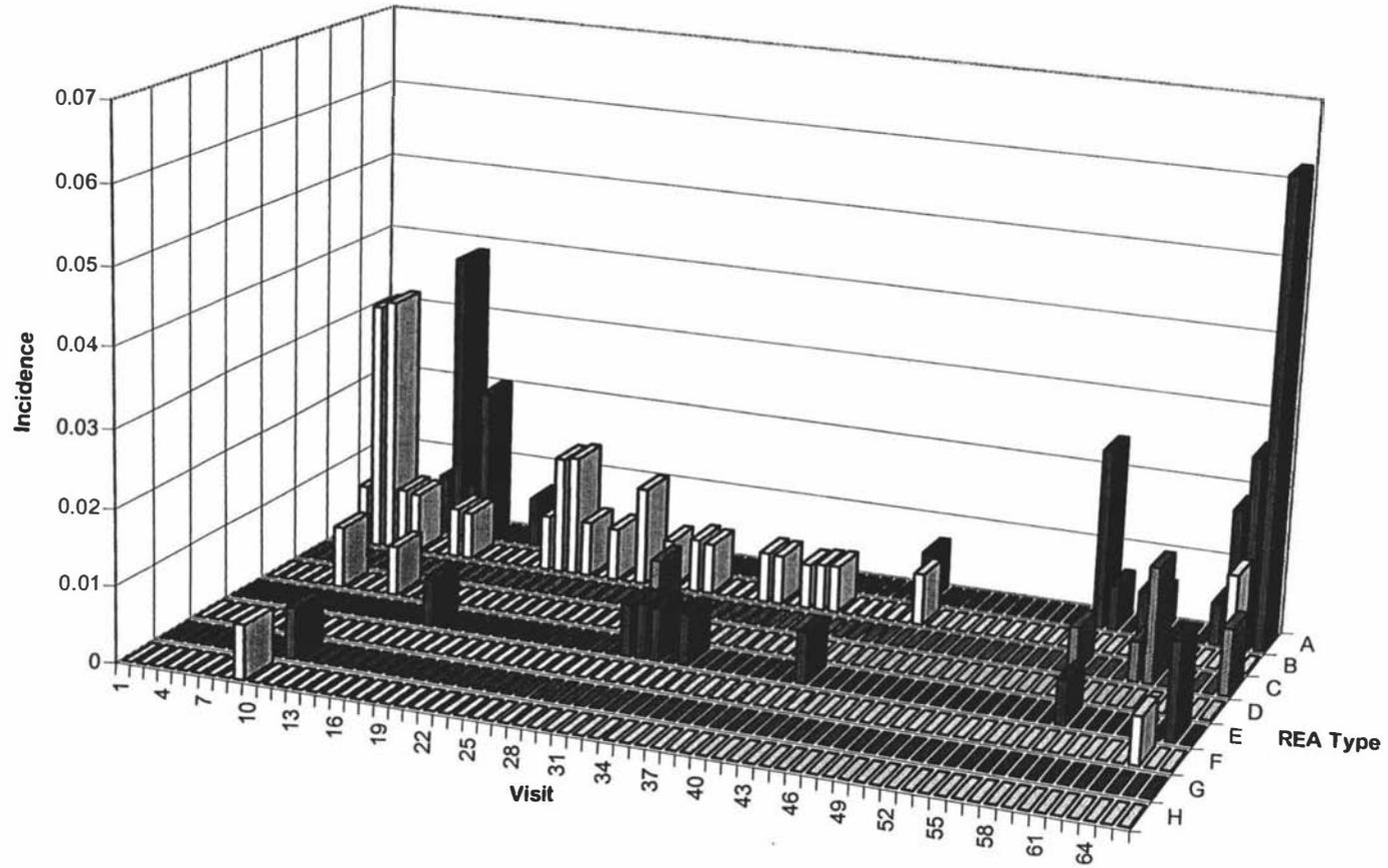


Figure 11-3. Temporal distribution of restriction endonuclease types of *M. bovis* isolates from possums on the study site. Incidence denominator derived from population estimate during the month in which the isolate was obtained

All of the possums with REA type C infection appeared to have fidelity to the northern section of the study site. This strain also exists on the properties surrounding the study site (Figure 11-2). These observations may actually document the establishment of this restriction type on the site, after possibly initially being introduced as a latent infection in Case 2418 (died in September 1991). This individual probably infected at least one other possum i.e. Case 2841. This latter case did not develop primary progressive disease, but was later subject to endogenous reactivation of tuberculosis and became infectious during the spring transmission period of 1993, during which she may have infected all of the latter cases known to be infected with this strain and which subsequently died in April/May 1994. Furthermore, if this sequence of events is correct, this suggests that the period from infection to death in this latter group of possums with what was presumed to be primary progressive disease, was approximately 7 or 8 months.

Although three cattle, two domestic deer and one ferret on the study site were infected with REA type C during the summer-autumn of 1993/94, it is not thought that the strain was common in areas peripheral to the study site, as there was only one possum with this REA type found during the termination in which 428 possums from the site and surrounds were killed. The occurrence of REA type C in a number of other species merely reflecting the unusual circumstances prevailing at that time (Chapter 5). The termination may have eliminated this REA type from the study site.

Restriction type D was found in two possums on the study site, but was also identified in 14 other mammals from other parts of the property and from immediate neighbours (Figure 11-2). Case 2450 was a 1.5 year-old juvenile male possum when first captured on the study site in May 1989. He unexpectedly died from tuberculosis caused by restriction type D, in January 1990. It is likely that this young male was pseudo-vertically infected and dispersed to the study site, only to die before showing evidence of having transmitted the infection to other possums. A juvenile female first captured on the study site in April 1989 was also infected with REA type D. This possum suffered an accidental death in September 1989, and was found at necropsy to have a closed axillary lesion only. She was accompanied by a pouch young which also died. It is possible that either of the above two possums may have infected each other as they were both trapped in the

valley floor area of the study site. In any event, it is quite likely that one or both were infected pseudo-vertically and were immigrants to the study site.

The majority (10) of the other animals infected with type D, which included five possums, two cattle, one pig (domestic), one goat (domestic) and one ferret, were located to the south and south-west of the property on which the study site was established, or the immediate neighbour on the southern side of the Castlepoint road (Figure 11-2). It is therefore probable, that the two possums on the study site which were infected with type D, may have acquired their infections in the south-western part of the property, a distance of at least 1.5 km from the study site. Of the other 5 animals infected with REA type D, three were cattle which had grazed in a number of locations on the study site property prior to infection being detected, one was a ferret captured on the study site in 1990 and another was a ferret captured in the neighbouring property to the north of the study site. Because of the large activity areas of the above animals, little can be said regarding the likely source of type D infection in them.

Restriction type E was only ever identified in twelve possums on the study site, and a small number of other species elsewhere on the same property i.e. four cattle, two wild pigs and one deer. These other animals were thought to have activity areas to the west of the study site. This restriction type was not identified on other properties in the area.

From the plot of the temporal occurrence of REA type E in possums (Figure 11-3), it could be speculated that the first occurrence of REA type E, Case 3620 which died in June 1990, was an index case which precipitated disease in the next five or six cases which followed. However, this is unlikely to have been true. Two of the next cases were yearling immigrants to the study site which were not present when Case 3620 died. Two others, although present at the time, had activity areas centred around the Middle and Queen St sections (Chapter 10) of the site, and were likely to have little interaction with the first case identified, which inhabited the valley floor area. However, transmission of infection to other possums by the “index case” cannot be totally excluded as she was likely to have been infectious during the autumn 1990 mating period, at which she was sexually active. The case identified in Visit 58 (Figure 11-3) also arrived on the study site after other known cases with REA type E had all died. At the termination another four cases infected with REA

type E were identified. These were all located in the Queen St. area, which suggests that this may have been closer to the major reservoir of this restriction type than elsewhere on the study site, and that this area may have “seeded” the study site with the earlier cases infected with this strain of *M. bovis*.

Restriction type F was isolated from only 3 animals. One of these was a male possum (Case 3747) which resided, and was regularly captured on the study site from October 1989, till his death from exposure/starvation in August 1994, at the approximate age of 8 years. Characteristic lesions were not present at necropsy, but *M. bovis* was isolated from culture of pooled lymph nodes. The only other recorded isolation of this REA type has been from a possum and a ferret, found at least 2 and 3 km, respectively, to the west (Figure 11-2). As this animal first provided evidence of infection when tested by LTA in February 1992, it is believed that this animal was a long-term latent carrier of infection (Chapter 9), incapable of shedding sufficient organisms to infect other possums in his activity area.

Restriction type G has also only ever been isolated from one possum on the study site, and has never been found elsewhere. This isolate came from a 6 year-old female (Case 3686) first captured in November 1989. She died from unsuspected tuberculosis in January 1990. At the time of death she was carrying a spring born pouch young which also died. Because of the age of this possum at death, it is likely that she would have been a long-term resident of the study site, and a latent carrier of infection acquired in another area. The infection was probably reactivated by the stressful environmental conditions prevailing around the commencement of the study (Chapter 11), but failed to spread to other animals, as her last offspring died, and there was little opportunity for transmission to cohorts at the stage of highest infectiousness (summer).

Restriction type H has only ever been isolated from one possum, Case 2741 which was initially captured on the study site in May 1989 as a 2 year-old female. A pharyngeal swab collected at first capture failed to provide an isolate of *M. bovis* following culture. However, this possum died from previously unsuspected tuberculosis in December 1989. She had a pouch young born in April 1989, which was last seen, as an independent juvenile, in December 1989. No spring-born offspring was observed. It is possible that during the period in which this possum may have been infectious, that she may have transmitted infection to her autumn

born offspring, which subsequently died from the disease. As she did not breed in the spring, the possibility of horizontal transmission is likely to have been limited.

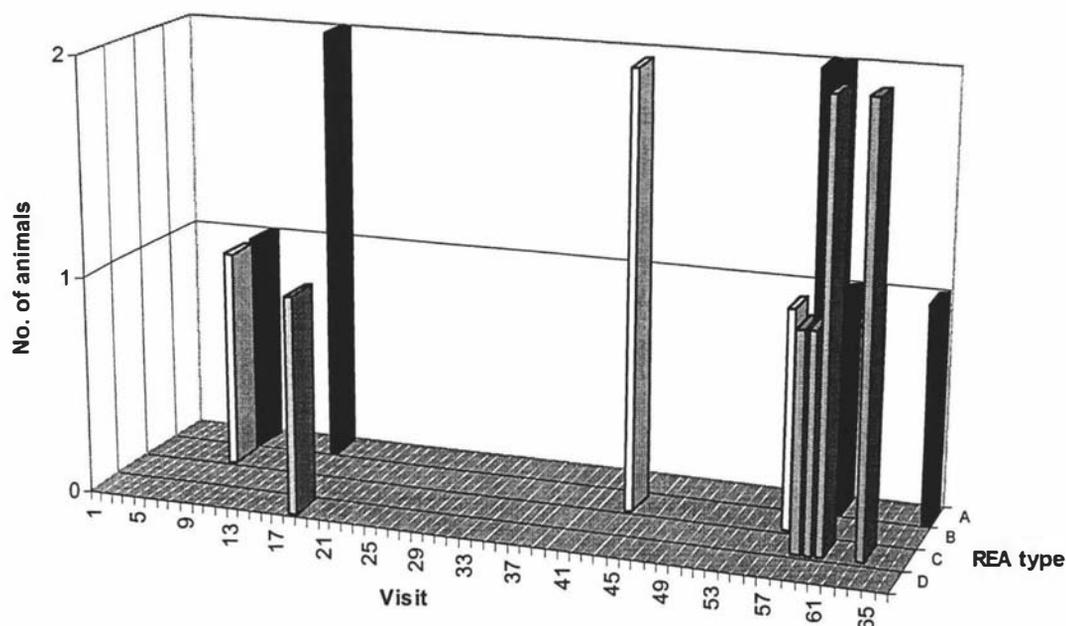


Figure 11-4. Temporal distribution of restriction endonuclease types of *M. bovis* isolates obtained from species other than possums (but including domestic deer and cattle) which inhabited the study site or immediately adjacent area

Comparison of the data presented in Figures 11-3 and 11-4 shows that the isolation of the REA types A, B, C, and D, mirrors the temporal occurrence of the same REA types in possums. Restriction type E, a moderately common strain in possums, is inexplicably absent from the isolates of other species taken from the study site.

Discussion

REA strain differentiation has proved to be a powerful tool for studying the epidemiology of *M. bovis* infection. It has allowed the identification of large and small scale clusters of apparently identical isolates and has allowed the tracking of patterns of disease transmission on and around the study site. However, conclusions reached in these studies are limited by the unknown proportion of unidentified infected animals which may have existed on the study site and environs and for which no REA data is available.

There is no evidence arising from the data (Table 11-I) to suggest that any restriction type is adapted to, or occurs exclusively in any particular host species, which supports the view that *M. bovis* has a broad host specificity. Although there are restriction types which occur in only one species, this cannot be construed to imply any specific host adaptation, as the number of examples of those restriction types is too limited to draw such conclusions.

The presence of multiple restriction types which appear to be closely related is testimony to the natural mutation of *M. bovis*. However, the restriction types appear to be relatively stable, as a number of the same types have been recovered from animals in the area over a 12 year period. This suggests that REA types were sufficiently stable over the time period involved in this study, to allow valid epidemiological conclusions to be drawn from the data presented. It should not be presupposed however, that the mutation rate is particularly slow, as the discovery of new restriction types may be a function of the number of isolates examined rather than the intrinsic rate of mutation. Although a new restriction type may be identified by only a single fragment line on one of the three enzyme patterns, it is unknown by how much, or where, the genome must change before the variant appears as a new restriction type. The phenotypic significance of the genetic differences identified by REA typing is presently unknown.

The fact that restriction types A and I are so widespread in the Wairarapa, and that multiple isolates were reported by Collins *et al.* (1986) would suggest that these strains may have been some of the earliest to establish in the Castlepoint area. As further REA types developed from the established parent strains their geographical distribution was initially limited, exemplified by restriction type E which has only been identified on or near the study site. As animals infected with these new variants became more common the geographic range of the strain extends to cover a number of properties, such as has occurred with type C, which has been found only on Waio station or immediately adjacent holdings. Some of the new restriction types may fail to persist if the effective transmission rate of infected hosts is insufficient to maintain the infection. Many restriction types, because of their localised distribution may also exist unrecognised until greater numbers of isolates are examined, such as has been the case with the wildlife around the Castlepoint

environs, where numerous restriction types have been identified, often in only one or a few individual animals.

The close geographic linkage between isolates of the same restriction type, in association with the limited activity areas of possums, suggests that each type is being maintained by possums in that area. The survival of particular restriction types in microhabitats for over 5 years on the study site suggests that the possum is a true reservoir host (Morris and Pfeiffer, 1995), that the host-parasite relationship is reasonably stable, and also that the organism has become possum adapted.

The close similarity between DNA fragment patterns of widespread New Zealand *M. bovis* isolates examined by Collins *et al.* (1986) suggests that all New Zealand restriction types may have had a common or closely related ancestors. This suggests that the transmission of infection from domestic stock to possums, which presumably occurred independently at multiple sites (Morris and Pfeiffer, 1995) must have occurred with restriction types which were closely related. This situation is most likely to have arisen from infection transmitted from wild red deer, of which there were only limited introductions from Britain in the late 19th and early 20th centuries. These red deer were distributed across the north and south islands following natural increase of the seedstock (Challies, 1990). It is quite conceivable that tuberculosis was present in the first deer introduced to New Zealand as these were derived principally from British deer parks, some of which have since been shown to be infected with tuberculosis. This is opposed to the likely scenario in cattle which would have been more varied in their source and time of introduction, and thus more likely to have introduced distinctly different REA types into the possum populations of the endemic areas. Areas such as Taranaki and Northland, which currently remain free of tuberculous possums, have traditionally been free of wild deer, an observation which supports the association between deer and the development of endemic areas. After possums first became infected, the mycobacteria may have undergone sufficient genetic change for numerous different restriction types to have emerged, often only distinguished by one band difference in a single restriction enzyme, as observed in this study and in the earlier reports of Collins *et al.* (1986) and de Lisle *et al.* (1995).

A particular restriction type is held in its own locality, probably over many years, by maintenance of infection in possums, although amplification of infection in

secondary hosts such as ferrets and deer, may also be important in some areas where possums are scarce. These wildlife reservoirs, of which the possum is probably the most significant, are capable of infecting inquisitive cattle and deer which come to investigate the moribund tuberculous creatures (Paterson and Morris, 1995; Sauter and Morris, 1995a). Sudden upsurges of cattle tuberculosis in an area are often associated with a single strain of *M. bovis*, even though more than one type is present in the same area (Collins *et al.*, 1988). This suggests that infection in the cattle is caused by one or a small number of the infected possums of that area, and that this is in turn related to the incidence spikes of particular REA types causing clinical disease in possums, as seen on our study site.

These studies support the hypothesis that there is not only significant spread of infection between domestic stock and wildlife, but also between various species of wildlife. However, although DNA typing data does not indicate in which direction the transmission occurs, the data does nothing to upset the hypothesis that the possum is the maintenance host of central importance, and that infection is likely to flow from possums to other hosts in most instances.

The data shows not only that outbreaks of tuberculosis with the major REA types of the study site were clustered in time, but also that the prevalence of the various REA types altered over time. This suggests that the dominant strains involved with disease in a locality are subject to temporal variation, with the most likely explanation for this phenomenon being natural variation in the opportunities for transmission presented to particular infected individuals. Persistence over time may be readily explained by the maintenance of subclinical disease or prolonged carriage of latent infection. This implies that the REA types which persisted on the study site were present continuously on or close to the study site, despite there being periods when they were apparently absent.

The strains which appear to have not persisted on the study site, such as types F, D, H, and G, probably failed to establish because there were too few infectious individuals, and these only capable of transmission during restricted periods, when the likelihood of infectious contact with cohorts is minimal i.e. outside of the breeding periods and aggregation at the pine trees in late winter. The high prevalence of infection with the dominant strains, types A and B, which existed in the population early in the course of the longitudinal study (Chapter 10) is also

likely to have precluded the possibility of superinfection with the newly introduced restriction types. There is sufficient reason to believe that the less common REA types, represented by one or two cases each, arrived with immigrant possums from other areas. The number of such incursions being inversely related to the distance from the cluster of that particular REA type. However, it is conceivable that types G and H which were not found elsewhere, could represent evidence of the emergence of new strains on the site, but which failed to persist.

Some possums are likely to be highly infectious and able to transmit infection to a number of other possums, providing the necessary circumstances prevail through the period of high infectivity e.g. a female with respiratory excretion and draining skin lesions which is in pro-oestrus/oestrus and mobbed by a number of males.

Of four REA types of which there were initially one or two occurrences (C, D, F, G and H), only one, type C, appeared to establish on the site. This may have been a serendipitous event, but could have been aided by the initial type C clinical case occurring in late 1991, when there may have been a greater number of new and fully susceptible possums entering the study site population, compared with the earlier period when the other possums infected with types F, G and H may have been infectious. Furthermore, this suggests that the establishment of infection in a population of possums is difficult, with success partially dependent upon force of numbers. The first infections to establish in New Zealand possum populations may thus have involved simultaneous infection of a number of possums in any one locality.

Although REA typing is the best method available for characterising the different genotypes of *M. bovis* existing in New Zealand, it is still a crude tool. It is unknown just how much genetic variation in a restriction type needs to occur before a new strain can be identified. The biological significance of genotype alteration, both within and between restriction types is completely unknown.

Any environmental heterogeneity that might lead to local increases in either host density or contact rates, and hence reduce the threshold density for disease transmission would act to enhance the probability of development of disease clusters e.g. in the forest pasture margins of the Hohonu Ranges (Coleman, 1988), or the river flats/scrub at Flagstaff Flat (Coleman *et al.*, 1994). At the study site

possums are less likely to aggregate on the southern side of the site due to the presence of only a few isolated pine trees, and a pine plantation located close by. Possums on the southern side do not need to come down to the valley floor to graze, as the hillside on which they den contains ample scattered pasture. On the northern side of the study site there are a number of isolated pines and a single row of large windbreak pines on the boundary, with none in the neighbouring property to the north (Chapter 10). Thus the presence of a few scattered pine trees and the windbreak on the northern side may provide sufficient cause for possum aggregation during the late winter, and may be the reason why several of the restriction types appear to have persistent clusters just off the northern side of the study site.

There has been no data arising from this study which challenges the view that possums are major reservoir hosts of tuberculosis in the Wairarapa. There is also no evidence to suggest that host adaptation of *M. bovis* has occurred, except in the case of possums, where they appear to be able to maintain clusters of individuals infected with particular restriction types in microhabitats for at least 5 year periods. The occurrence of newly introduced restriction types into these endemic clusters has made possible observations on the epidemiology of infection, including the documentation of the occurrence of latent infections, duration of primary progressive disease in newly infected possums and the likely occurrence of post-primary reactivation of tuberculosis.

Acknowledgments

I thank R.W. Maunsell, owner of Waio Station, for the use of a portion of his property. I am also indebted to Ron Goile, Donna Lewis, and others who have so ably assisted with the Castlepoint longitudinal study over the years. For the submission of specimens for necropsy by the Castlepoint farming community, I am also indeed grateful. For the establishment of the study, and the continuing conduct of the research prior to my period of study site supervision, I am indebted to Dirk Pfeiffer and Ron Jackson. I am also very thankful for the field assistance, over a number of years, of my colleague Carola Sauter. I am also grateful for the execution of, and advice regarding the REA typing by the staff at AgResearch, Wallaceville, particularly Geoff de Lisle, Gary Yates and Des Collins. Free access to historical data on *M. bovis* REA types of the Wairarapa was also kindly given by

Geoff de Lisle, and Garth Pannett, MAF, Masterton. Financial support from the Animal Health Board is gratefully acknowledged.

The PhD candidate was responsible for managing the Castlepoint study site from July 1993 through till April 1996. The majority of the possum necropsies, and all of those conducted on other species during the period of supervision were conducted by the candidate. Data collection and storage (with assistance) through this period, and subsequent analyses has been carried out by the candidate.

CHAPTER 12

GENERAL DISCUSSION

General Discussion

Although it could be considered that the scope of the research undertaken in this study was rather broad, it has provided a unique opportunity to study various aspects of the natural infection with *M. bovis* in a wide range of species, both domestic and wild. Although the disease manifests itself differently in each of its hosts, this exceptional opportunity has provided me with an holistic view of the disease and allowed me to develop robust hypotheses regarding the transmission of infection and pathogenesis, applicable to all species. This would have been difficult to achieve if the research had been more focused, and covered a smaller range of hosts. Thus, what could have been considered a weakness in the overall methodology, has been converted into a strength, for which the results of the research hopefully bear witness.

Host status

The differences in the ability of various species to become reservoir hosts broadly stems from issues of host susceptibility, from the existence and scale of different intraspecific transmission pathways, duration of the infection and infectious states, and from behavioural factors which determine whether the potential of specific transmission pathways can be expressed. In badgers, Anderson and Trehwella (1985) have suggested that pseudo-vertical transmission, duration of infectiousness, existence of carriers, social structure and behaviour are all likely to be important in the maintenance of infection.

Susceptibility

To be capable of maintaining disease in their own free-ranging populations, a species must be susceptible to both infection and disease following contact with *M. bovis* under field conditions. Both within and between species there is considerable variation expressed in the susceptibility to infection. Innate resistance, mediated by non-specific effector mechanisms may arrest the growth of mycobacterial pathogens without any measurable immunological response being generated and without the survival of any bacilli. Other infected individuals may develop specific cell-mediated immunity after exposure to infection and may continue to harbour viable *M. bovis*, whilst a minority develop primary progressive disease. The route of bacillary acquisition, numbers of bacilli and the nature of the antigen-presenting

cells initially handling the infectious inoculum, may all influence the type of response elicited by the T cells. In addition, cytokines produced by non-specific bystander cells, possibly modulated by physiologic stressors, may provide an environment that favours development of either T-helper 1 or T-helper 2 responses (Kemp *et al.*, 1996), which in turn may determine the outcome of the initial infection.

At the resistant end of the disease spectrum, are species such as rats and mice which are innately insusceptible to tuberculosis, and are thus unlikely to maintain viable organisms in their tissues, nor excrete sufficient bacilli to infect other conspecifics. Next in line in increasing susceptibility are species such as stoats, hedgehogs and goats. In these animals, many are likely to become infected by contact with infected possums. Most appear able to shrug off the infection after developing minimal or no gross lesions, whereas a few will develop severe disease. Next in order of susceptibility may be deer, sheep and pigs, each of which are capable of developing severe disease in a substantial proportion of the infected population, but under low-stress, or low challenge conditions, infection normally accounts for few deaths. Both possums and ferrets appear to be quite susceptible to infection and disease development. Rabbits (and possibly hares) are probably the most susceptible to severe disease following successful introduction of infection, but under ordinary circumstances it seems that they are unlikely to receive an infectious dose of *M. bovis*.

Transmission pathways

For animals to become reservoir hosts of tuberculosis, such as man, cattle, farmed deer, possums and badgers, the animals typically need to be able to shed the bacilli freely from lesioned sites for extended periods, with the more potential routes of excretion and scale of excretion, the more likely the species is to become a reservoir host. All the well recognised maintenance hosts of tuberculosis commonly develop extensive respiratory involvement, with the potential for infectious aerosol generation. In addition, there is the likelihood of excretion by other routes e.g. via suppurating sinuses especially in the case of deer, badgers and possums, and also from saliva, urine, milk, (and *in utero* in the case of cattle). However, animals such as deer and sheep which can develop extensive respiratory involvement, may be poor generators of infectious aerosols, as coughing is not a clinical feature which

accompanies pulmonary disease to the same extent as in cattle or humans, which are believed to be successful disseminators of infectious aerosols.

Draining sinuses are particularly likely to be a source of transmission to other deer, as is saliva following bacillary shedding from the oropharyngeal tonsils. There appears to be little opportunity for transmission *in utero*, via milk, faeces and urine in deer. The ferret also seems to be particularly limited in its means of transmission, such that spread in saliva during bite wounding would seem to be the only common transmission pathway between cohorts, thus making it unlikely for this species to be a reservoir host.

Pseudo-vertical transmission in possums and badgers may also be of considerable importance in maintaining infection in an area, and for the dispersal of disease with emigrants to new localities. However, pseudo-vertical transmission appears to be only a rare event in ferrets and deer, with no evidence to support its occurrence observed in the current studies.

In pigs, the results suggest a possible role for pseudo-vertical transmission, excretion from suppurating sinuses, from the respiratory tract in active generalised cases, and from the pharynx in animals shedding from the tonsils. However despite these transmission mechanisms being available, it would seem that once the load of *M. bovis* in the environment is reduced, the number of generalised cases, and hence potential bacillary excretors will fall, thereby ensuring that the feral pig will fail to maintain the disease in the absence of other infected hosts.

Duration of infection/infectiousness

To be an ideal maintenance host several criteria should be fulfilled. An individual should be able to survive for long periods in the infected and infectious states, and infected adults should be capable of reproducing, with the production of viable young.

At one end of the scale are rabbits, which once infected will virtually all invariably die within months. Although urinary and respiratory routes of excretion are available, the infectious wild rabbits will probably die before sufficient infectious contacts can be made, and they are thus probably incapable of becoming reservoir hosts.

At the other end of the continuum of longevity of infection, are humans, which are capable of being infected with *M. tuberculosis* for a lifetime. Only a small proportion of human infections progress to clinically active tuberculosis, and the probability of progression may be as low as 10% over the lifetime of an individual (Rieder *et al.*, 1989). It was demonstrated in the 1950s that most newly diagnosed cases of tuberculosis in humans arose from a pool of individuals in which the infection had been quiescent for a variable period of time and not from people with primary progressive infection (Rieder *et al.*, 1989).

The relationship between the number of infected possums without gross lesions, and those with gross lesions, a ratio of 1:2, apparently no matter what the prevalence of disease, is similar to the situation observed in red deer, ferrets and badgers (Gallagher *et al.*, 1976), in which it is believed that approximately one third of all infected animals are also without gross lesions at necropsy. This apparently constant ratio, implies the development of an equilibrium between various states of infection and suggests that in some cases the bacilli either die, decrease in abundance or become dormant, following a period of infection, such that culture of the predilection sites fails to isolate the organism. In both deer and ferrets (unpublished data), there is a tendency with increasing age for infected animals to be gross lesion free, which suggests that the lesions acquired earlier in life have regressed and/or that susceptible individuals have died, with those remaining having kept the infection in check and have not been subject to primary progressive disease.

Bacterial persistence in the dormant state, and endogenous reactivation are believed to be one of the crucial epidemiological features of human tuberculosis, without which, the disease probably would not exist (Grange, 1992). This is also likely to be true of *M. bovis* infections in animal reservoir hosts, where the endogenous reactivation of disease occurs not on a scale of years or decades, as in humans, but on a time scale appropriate to the longevity of the host, precipitated by environmental stressors at a population level, and by other factors, such as intercurrent disease in the case of individuals. Infection with the tubercle bacillus is the necessary cause of disease, but a breach of cellular immunity is the independent sufficient cause of disease. Certainly the apparent frequent occurrence of post-primary tuberculosis in man, probably possums, and possibly deer, in association with factors that weaken the immune defence mechanisms suggests that host

immunological, as well as adverse metabolic, factors contribute to the maintenance of the state of dormancy in tubercle bacilli.

Grange (1992), on the subject of bacillary dormancy, states that “Our attempts to understand the phenomenon of persistence have been limited by the fact that we are so used to thinking of the mycobacteria as being rod-shaped, acid-fast, non-sporing bacteria with simple life cycles of binary fission that, as a consequence, we generally deny the possibility of more elaborate life cycles involving very slowly growing stages, or resting forms developing in response to environmental stress.” Several distinguished early workers, including Calmette, believed that there was a virulent filterable form of the tubercle bacillus, which was not microscopically detectable, but others denied its existence. When it was demonstrated that up to 10,000 bacilli per ml of tissue must be present before acid-fast bacilli could be visualised, there was no longer a need to postulate the existence of variants other than the acid-fast form. Khomenko (1987) found the existence of spherical forms, up to 20 times smaller than bacilli, in the pulmonary tissues of patients undergoing anti-tuberculous drug therapy. He postulated that these forms represented one of the lesser known forms of bacterial ontogenesis, and are the natural response of mycobacteria to adverse chemical or physical factors. Such forms probably occur in all species capable of mounting a successful immune response to mycobacteria.

Although Clifton-Hadley (1996) believes that in reservoir hosts, the disease should not significantly affect either population numbers or structure, there is some evidence from possums to suggest that population density may be significantly reduced by outbreaks of tuberculosis. It would seem, that so long as there is a significant proportion of latently infected survivors in a residual population, the disease will persist, with further epidemics often initiated by synchronous reactivation of tuberculosis in the carriers, induced by some event, possibly a stressor of some kind.

Behaviour/Social structure

Apart from being capable of developing disease following infection, and being able to excrete bacilli, the reservoir host must be able to ensure, that by their behaviour, there will be sufficient infectious contacts made with cohorts to maintain the disease in the population.

Badgers form social groups, share setts, wound each other and sleep in close contact. These social arrangements provide good conditions for both the survival of bacilli and for oral or aerosol transmission of infection, which would greatly facilitate the dissemination of infection within a social group. In possums, an animal without exclusive activity areas and with little social contact, the opportunity for direct transmission is more limited. Transmission is likely to occur between mother and offspring during the rearing period, between adult males and females during courting and mating, and during simultaneous den sharing (where this occurs), and at food sources where focal aggregation and agonistic encounters occur.

Although wild deer are social creatures, especially at high densities, intimate interactions are probably principally restricted to mother-offspring associations, mating and agonistic encounters during the rut. This limits the opportunities for direct transmission, especially given the apparently restricted generation of infectious aerosols from those with pulmonary tuberculosis. In the wild it has also been observed that sick individuals (i.e. those which are likely to be highly infectious) segregate from others, presumably to escape victimisation, to die in isolation. This behavioural characteristic is not able to be expressed in farmed deer, and probably forms the basis for the difference between wild and farmed deer patterns of tuberculosis. Farmed deer often suffer severe outbreaks associated with individuals afflicted with suppurating sinuses, and they can thus be clearly defined as reservoir hosts of tuberculosis. However, the status of free-ranging deer is less clear, although local and overseas evidence of persistence of *M. bovis* infection in extensively managed or wild deer, albeit at low prevalence, would suggest that free-ranging deer may in at least some situations, act as low prevalence maintenance hosts for tuberculosis.

Ferrets usually live as isolated individuals with little social interaction, except when rearing young, or during the mating period. There is at present insufficient evidence to rule the ferret out as a reservoir host, however the bulk of the evidence would suggest that the only common means of intraspecific transmission is by bite wounding. Given the current evidence, which includes the scarcity of transmission opportunities, it would seem wisest to classify ferrets as spillover hosts, with a role in disease amplification only.

Species roles

The epidemiology of *M. bovis* infection in New Zealand is very complex, and it now appears that over the last three decades the bovine tuberculosis control campaign has been successful in changing the cattle and farmed deer populations from maintenance hosts to spillover hosts for wildlife infection. Wildlife infection appears to be principally maintained by possums (Figure 12-1), with the area affected still expanding, but becoming increasingly difficult to accurately define. The difficulties in identifying infected possums in low density possum habitats or new areas of endemic infection may relate to the development of a better host-parasite relationship in possums, in which latent infection, without visible gross lesions, may be increasing in frequency.

Wild deer should be principally considered as spillover and amplifying hosts for tuberculosis, with most infection acquired by bold and inquisitive individuals which closely, and perhaps vigorously, investigate moribund tuberculous possums. However, they have the potential for a low level of disease maintenance which will increase in importance as possum tuberculosis control methods become more successful. Both tuberculous farmed and wild deer are capable of introducing disease into sympatric possum populations causing the development of new disease foci. Their role in this regard must not be overlooked. Indeed, the original infection of possums in endemic areas may have developed from the molestation of possums by tuberculous deer. Possums may also have contracted tuberculosis through carcass feeding when protein deficient, in areas where drought may have been devastating the countryside, and where wild deer were at high density and perishing. One such situation in the Wairarapa in the late 1940's, has been reported by Thomas *et al.* (1993), where starving possums abroad in daylight, were seen to feed on the carcasses of dead deer. In this circumstance, when densities of deer were high and suffering from environmental stress, the prevalence of tuberculosis may have been substantial, and sufficient to transmit infection to possums. Possums are now well recognised to incorporate flesh in their diet (Brown *et al.*, 1993), and are readily captured in cages or traps with meat lures.

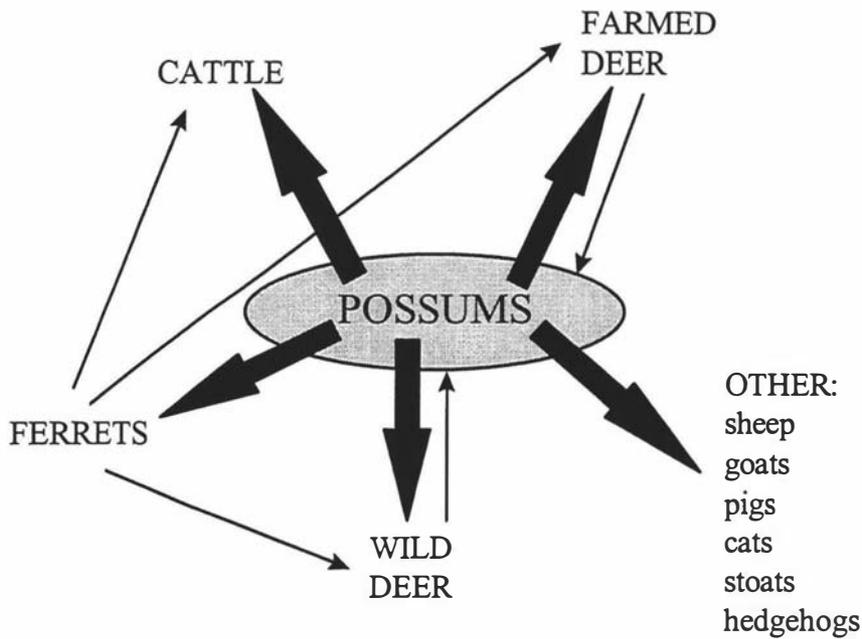


Figure 12-1. Species currently involved in the epidemiology of *Mycobacterium bovis* infection in New Zealand. Arrows indicate the magnitude and direction of the principal transmission pathways (diagram adapted from Pfeiffer, 1994)

Of the wild carnivores found in New Zealand, the disease persists at high prevalence only in ferrets, and is probably maintained principally by ingestion of tuberculous carrion. Of the amplifying hosts, they are probably the most significant, as they, like possums are subject to investigation by inquisitive livestock when found moribund on pasture. They are also one of the more useful indicator species, which can be used to establish whether *M. bovis* infection is present in local wildlife. Although often not numerous, the high prevalence and long duration of infection, ease of trapping, handling and diagnosis have made them ideal for this purpose. Wild pigs are also useful indicators of the presence of *M. bovis* in an area, but the potentially large home ranges may make pin-pointing the source of infection difficult. In hedgehogs infection also appears to arise from scavenging. The moderate prevalence of tuberculosis in these animals, combined with their small home ranges may allow them to be used successfully in wildlife surveys to pinpoint the locality in which tuberculous possums have died. However, those which become diseased are likely to develop only minor lesions which resolve over time, making infected individuals difficult to detect, especially when tuberculous possums have been absent for a lengthy period (R. Gorton pers. comm.). Of the other species which have been shown to be capable of carrying

infection, none is likely to play a significant role in the epidemiology of *M. bovis* infection in New Zealand.

The hypothesised ability of individual possums to maintain endogenous disease, without demonstrating recognisable gross lesions at necropsy, may be the cause of new foci of endemic disease appearing in the apparent absence of possums with gross lesions identifiable at necropsy. In these areas, e.g. the lower Rangitikei, and North Canterbury areas a small number of infected possums, subject to endogenous reactivation of tuberculosis, or primary progressive infection following transmission from an index case(s), may be largely responsible for the occurrence of skin test reactors in domestic stock, and for the presence of infected ferrets in the area (Figure 12-2). As wildlife cross-sectional studies in these areas are usually conducted retrospectively, with respect to the identification of bovine reactors, the spike in the incidence of clinical disease in possums has passed, and lesioned cases almost (or are) impossible to find. However, infected ferrets, a good indicator species, are frequently identified, and erroneously blamed for the initiation of the outbreak of tuberculosis in the domestic stock. By contrast, in established endemic areas, with moderate to high possum densities, there will always be sporadic cases of tuberculosis amongst possums which will provide a continuous trickle of bovine cases.

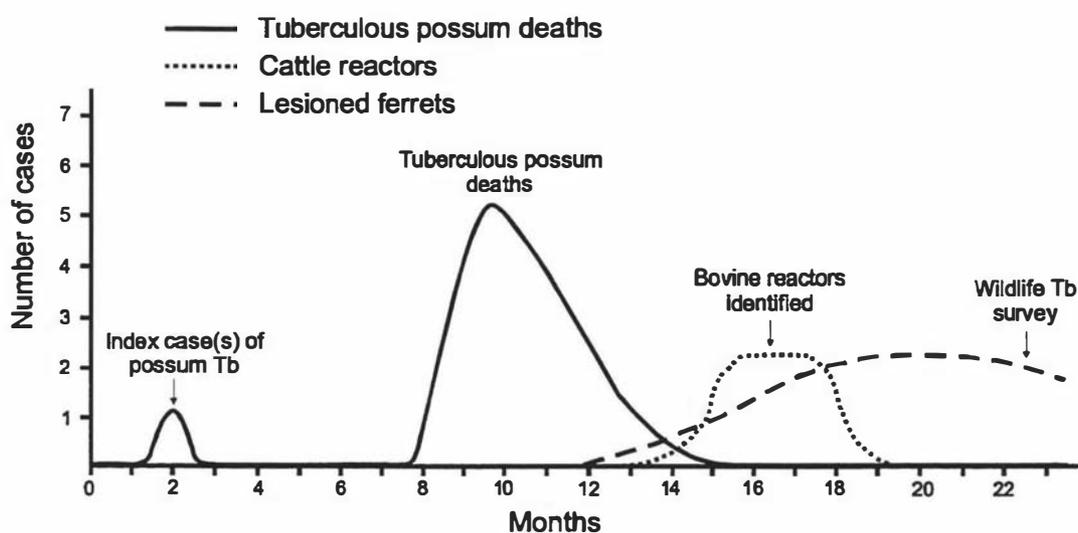


Figure 12-2. Schematic representation of selected epidemiological events surrounding a new outbreak of tuberculosis in possums

One wild card which exists in the epidemiology of tuberculosis, is the role of strain variation in changing the expression of disease in a host, and potentially allowing what was a spillover host, to become a reservoir host. This may have occurred in the goat strain which occurs in Mediterranean countries. Historically, in other parts of the world, it appears as though goats infected by bovine derived *M. bovis* isolates have suffered a low grade infection which failed to become endemic in goat populations, whereas in the Mediterranean, strains of *M. bovis* are now present which are highly pathogenic for goats, the reservoir host of these strains (Gutiérrez *et al.*, 1995). A similar phenomenon may have occurred in Britain, where a new *M. bovis* variant has been found in a series of cats (Gunn-Moore *et al.*, 1996; Blunden and Smith, 1996).

Mucosa-associated lymphoid tissues

Koch (1886) observed that tuberculosis “appears under a different aspect in each species” and Thoms and Morris (1983) also thought it unwise to make generalisations regarding the pathology between different species. These studies would confirm these remarks, but nonetheless there are aspects of the pathogenesis common to all of the susceptible hosts. The involvement of the lymphoepithelial tissues, in the uptake and excretion of mycobacteria is one such common thread, which has emerged.

The lymphoepithelial tissues must be regarded as “immunologically privileged” sites which are not regularly subject to the vigorous inflammatory responses which characterise tuberculosis infection at many other sites. Thus the oropharyngeal tonsil, nasopharyngeal tonsil and Peyer’s patches, although primary infection sites, which excrete bacilli and disseminate infection to other sites, often undergo no change in appearance which might alert the observer to their true, and hitherto, overlooked role in the disease process.

Directions for future research

A study of mucosal immunity, mycobacteria and the lymphoepithelial tissues is likely to provide a fruitful area of research. There is ample basic research required on the interaction of mycobacteria and these lymphoid sites, which should include: mechanisms of mycobacterial uptake; excretion and dissemination of bacilli; responses to size of infecting dose; duration of mycobacterial residence in MALT, and the nature of immune responses generated. This research will have useful spin

offs in terms of better understanding the pathogenesis of infection, and may ultimately improve the efficacy of vaccination techniques.

The existence and nature of unusual forms and life cycles of mycobacteria is a field wide open to investigation. The available epidemiological evidence in possums should neither be rejected nor accepted uncritically, but subjected to rigorous evaluation by the use of state-of-the-art technology, which should include electron microscopy, immunohistochemical, and molecular biological techniques. The development of PCR as a diagnostic tool for use on wildlife blood samples may prove very useful. Experiments to force "latently" infected animals to reactivate their infections, through the administration of glucocorticoids should also be considered.

On the assumption that stressors are capable of inducing disease progression in possums, a better definition of the causes of such stress are required. Is heat or water stress important in possums? Is it weight loss, or a particular nutritional deficiency, which is important in reducing resistance to tuberculosis in possums? What climatic events can be associated with an increase in tuberculosis prevalence in livestock in endemic areas, and hence putatively with reactivation of disease in possums? What is the importance of intercurrent disease, especially of the kidneys, in causing disease progression in possums? These are just some of the questions for which answers are required.

Examination of available data on possum populations should be undertaken to confirm whether biannual breeding of possums is a significant risk factor for endemicity of tuberculosis. Research by management also needs to be conducted to assess whether habitat alteration, to remove putative focal food sources, will reduce the prevalence of tuberculosis in possums and domestic stock. With a better knowledge of the factors which precipitate disease, predictive models should become more accurate, and on-farm management options more refined.

Given that red deer may be reservoir hosts of *M. bovis* it would seem prudent to continue investigations into the epidemiology of tuberculosis in cervids. Clarification of the role of other species of wild cervids in New Zealand is required. Isolated *M. bovis* infected deer populations, from areas in which tuberculosis is not endemic, need to be found, to accurately establish the prevalence of infection and

the host status of wild deer. Uninfected red deer populations, outside of endemic areas also need to be identified to satisfactorily test the hypothesis that infected possums are responsible for maintaining a high prevalence of infection in wild deer.

Despite sheep being one of the most common species of mammal in New Zealand, little useful data exists on the prevalence of tuberculosis in sheep flocks in endemic areas, nor of the risk to other livestock posed by the presence of tuberculous sheep. Despite clarification of these issues being of suspect agro-political merit, the answers may ultimately be important to disease control and eradication programmes.

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Appendix I: Critical necropsy technique for deer used during these studies

Requirements:

Necropsy site

Where possible, necropsies were performed at Massey University post mortem examination room. Here it was more convenient, safer, easier to clean up and dispose of carcasses. Where the task was done on farm, then the site used was exposed, dry, well drained, handy to facilities and fenced from stock access (at least for the short term). Carcasses were disposed of in a nearby pit from which predators and scavengers were excluded. Contamination of people and the environment were kept to a minimum by wearing overalls, disposable gloves and rubber boots and attending to routine cleaning procedures.

Equipment

Virkon™ at 2% strength was used as the preferred disinfectant as it is less noxious than the phenolic alternatives. Rinsing instruments in water to remove the Virkon was necessary to avoid contamination of culture samples with disinfectant

A hot water sterilizer, typically a billy of boiling water over a gas burner, was used to decontaminate instruments. Instruments were immersed in the boiling water for 30s or longer. This was a necessary and rapid way to prevent cross-contamination between tissues and animals. Care was taken to avoid accidental contamination of the hot water with disinfectant.

White PVC plastic butchers trays were found ideal for laying out organs and lymph node collections for examination. Pre labelled paper sheets with a checklist of lymph nodes for examination and spaces for laying out the important nodes were very helpful to compare the size of node pairs and to ensure that all sites were properly examined. Sample sheets used are appended below.

Necropsy procedure

General comments

It was important when killing the deer to ensure that aspiration of blood was avoided as this prevented the lungs from collapsing and hindered the examination. Ensuring that the head is lower than the chest after cutting the throat or bleeding-out without cutting the trachea will prevent blood aspiration.

Many deer shot by hunters, were presented incomplete, and in these instances a modification to the examination procedure presented below was carried out, depending upon the portions which were available.

A thorough necropsy on a whole carcass often took up to 2 hours for each animal, thus making it essential to hold portions of the carcass at a comfortable working height. Inspection of nodes and other tissues was carried out under good light conditions so that fine detail of the nodes was visible. Each node examined was sliced into 3 mm sections (maximum), such that the smallest of lesions could not be overlooked. This was done with a sharp knife or scalpel, usually after removal to the labelled paper sheets on plastic trays.

Swabs of the nasal cavity, and the tracheal mucosa were taken at the start of the necropsy. A pharyngeal swab was not collected till after the head had been removed and the tongue reflected back. The area near, but not including the tonsillar fossae, was wiped by the swab. Unfortunately this area often was contaminated by quantities of ingesta or rumen contents, the presence of which will have decreased the sensitivity of culture.

Immediately after the abdominal contents were removed the bladder was inspected and a urine aspirated into a vacutainer tube if possible. Deer which had been carried suspended by the head under helicopters often had empty bladders, as the weight of abdominal contents expressed the urine whilst in transit. Several faecal pellets were squeezed into a pottle from the cut section of the rectum.

Where possible samples of the following tissues were collected for individual culture: Nasopharyngeal tonsil, pooled portions of the oropharyngeal tonsil, pooled portions of the medial retropharyngeal lymph nodes and pooled sub-samples of the left tracheobronchial, cranial tracheobronchial and the caudal mediastinal lymph nodes.

Sub-samples of tissues for mycobacterial culture were carefully selected to include areas likely to contain viable bacilli. Sample size was kept to 5 or 6g so that the culturing laboratory would find it unnecessary to discard any selected material. Tissues were placed in sterile plastic pottles which were then chilled or frozen as soon as possible. If frozen, samples were not allowed to thaw or refreeze before arrival at the culturing laboratory.

The following discussion briefly describes the procedure used for examining a carcass, section by section, with appropriate comments on technique where this varies from routine necropsy methods. Each section starts with a list of nodes (and synonyms) or sites that were examined thoroughly if available.

Head

Sites: Parotid lymph node (ln.), Mandibular (submaxillary) ln., Lateral Retropharyngeal (atlantal) ln., Medial Retropharyngeal (retropharyngeal) ln., Oropharyngeal (palatine) tonsil, Nasopharyngeal tonsil (adenoids).

The head was removed from the body for examination. It was severed through the atlanto-occipital joint as far caudally as possible leaving the larynx and pharynx intact to facilitate the location of the lateral retropharyngeal lnn.

The mandibular nodes were removed first, and then the parotid nodes together with the dorsal half of the parotid salivary gland in which it is embedded. The lateral retropharyngeal nodes (can be several on each side) are found in behind the mandibular salivary gland. The tongue was reflected and cuts made through the most rostral hyoid joint to continue exposure of the medial retropharyngeal nodes deep in the pharynx. The oropharyngeal tonsils were best removed with the surrounding section of pharynx, cutting close to the jaw to avoid leaving part of the tonsil in situ. The nasopharyngeal tonsil is a single lymphoid structure of thickened and folded mucosa at the caudodorsal end of the nasopharynx. This was always removed for culture unless destroyed by shooting. Sub-samples of the oropharyngeal tonsils always included any crypts containing caseous or mucoid exudates or any other suspicious lesions.

Thorax

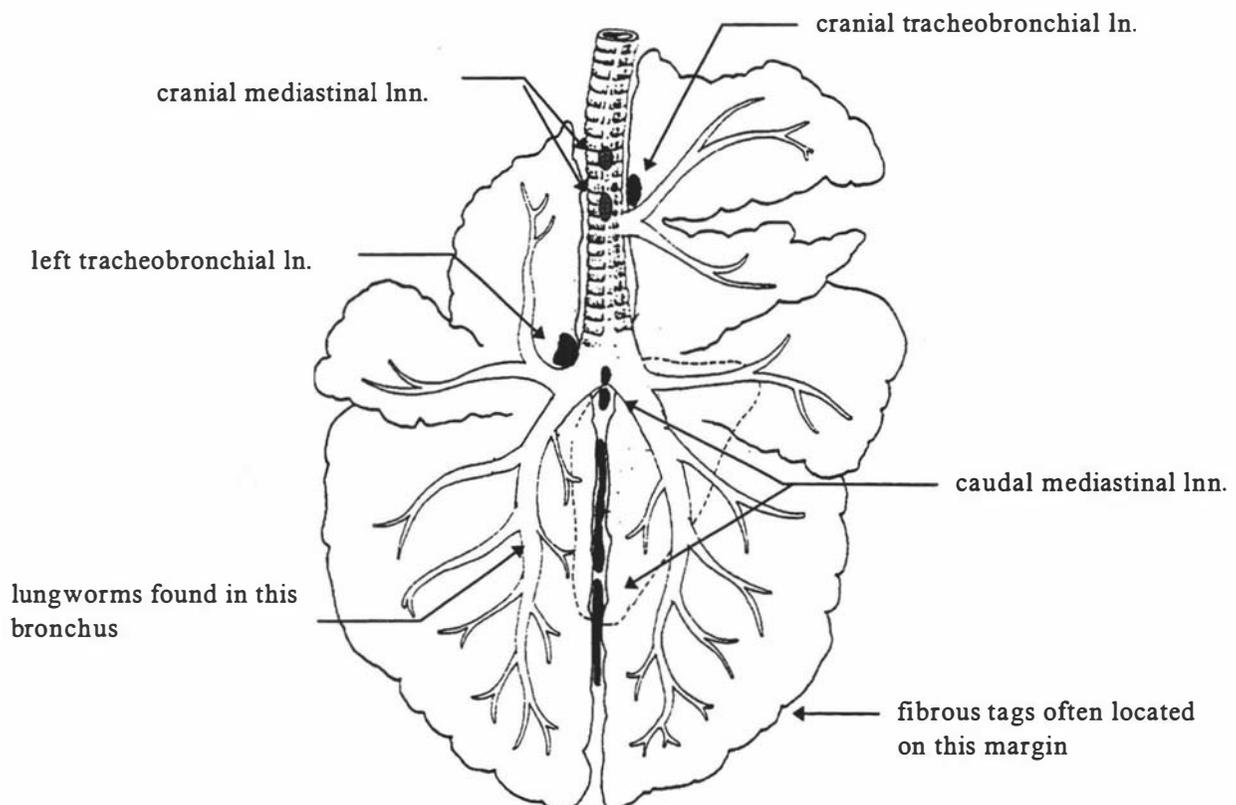
Caudal mediastinal ln., Left tracheobronchial (left bronchial) ln., Cranial tracheobronchial (apical) ln., Lung, Cranial mediastinal lnn., Sternal lnn., Aortic lnn., Right tracheobronchial ln., Heart.

The best view of the thorax was obtained by removing one side of the rib cage with large pruning secateurs. This gives a clear view of the thoracic contents with minimal disturbance or blood splash, and also allows easy detection of pleural adhesions. A faster and reasonable alternative often employed was the removal of the entire pluck through the diaphragm. After slicing around the tracheal and oesophageal attachments at the thoracic inlet, the diaphragm was cut to expose the

caudal chest. Mediastinal attachments were severed dorsal to the aorta to preserve the caudal mediastinal nodes intact. Note was taken of any adhesions during the removal of the pluck.

The two halves of the lungs were examined by dissecting them away from the mediastinum and severing the major bronchial attachments to the trachea. The lungs were laid out on trays for initial superficial palpation, followed by cutting along the main bronchus supplying the diaphragmatic lobes, checking closely for presence of lungworm and associated lesions. The lobes were then sliced into 2.0 - 2.5 cm wide strips so that the entire parenchyma could be palpated and small nodules located. Attention was then directed to the bronchial and mediastinal lnn., the locations of which are shown in Figure A-1. The left tracheobronchial node lies under the aorta as it emerges from the heart. The cranial tracheobronchial node lies just in front of and/or below the bronchus which supplies the right cranial lung lobe (the position and number of this node is variable). A right tracheobronchial node can sometimes be found caudal to this bronchus. Cranial mediastinal nodes are located ventral to the trachea.

Figure A-1. Diagram of deer lung showing major lymph nodes and other sites of interest.



Abdomen

Abomasal Inn., Duodenal In., Jejunal Inn. (chain), Ileocaecal Inn., Hepatic Inn., Liver, Kidney, Spleen, Rumenoreticular Inn., Omasal Inn., Rectal Inn., Renal Inn.

In the field the abdominal viscera were rolled onto a plastic sheet to keep it clean during the examination. Isolated organs, such as spleen, liver and kidneys, were placed in the trays for close inspection and sectioning. Because of the great length of the mesenteric chain it was very tedious to remove all nodes for checking on the paper sheets. Fine systematic sectioning in *situ* normally proved satisfactory.

Body

Superficial cervical (prescapular) In., Subiliac (precrural) In., Superficial inguinal In., Popliteal In., Medial iliac (internal iliac) Inn., Lateral iliac In., Axillary In., Cervical chain Inn., Ischiatic In.

The body nodes were best examined on the paper sheets to ensure that all were present and to assist with size comparisons between the left and right.

Examples of lymph node work sheets

On the next pages are the three sheets with tabulated lymph nodes which were used to aid the examination of the lymphatic system. These are also suitable for use with other ruminants.

MANDIBULAR	LEFT	RIGHT	HEAD
PAROTID			
LAT. RETROPHARYNGEAL			
MEDIAL RETROPHARYNGEAL			
OROPHARYNGEAL TONSIL			
NASOPHARYNGEAL TONSIL			

SUP. CERVICAL (prescapular)	LEFT	RIGHT	BODY
SUBILIAC (precrural)			
POPLITEAL			
SUP. INGUINAL			
MEDIAL ILIAC (Group)			
Others that can be checked: Lat. Iliac, Axillary, Cervical chain, Ischiatic.			

CAUDAL MEDIASTINAL	THORAX	ABOMASAL	ABDOMEN
LEFT TRACHEOBRONCHIAL	DUODENAL		
CRANIAL TRACHEOBRONCHIAL (apical)	ILEOCECAL		
CRANIAL MEDIASTINAL	HEPATIC		
OTHERS: Sternal, Aortic, R. Tracheobronchial	OTHERS: Rumenoreticular, Omasal, Rectal, Renal		
JEJUNAL			

Appendix II. Population estimate of possums on the Castlepoint study site

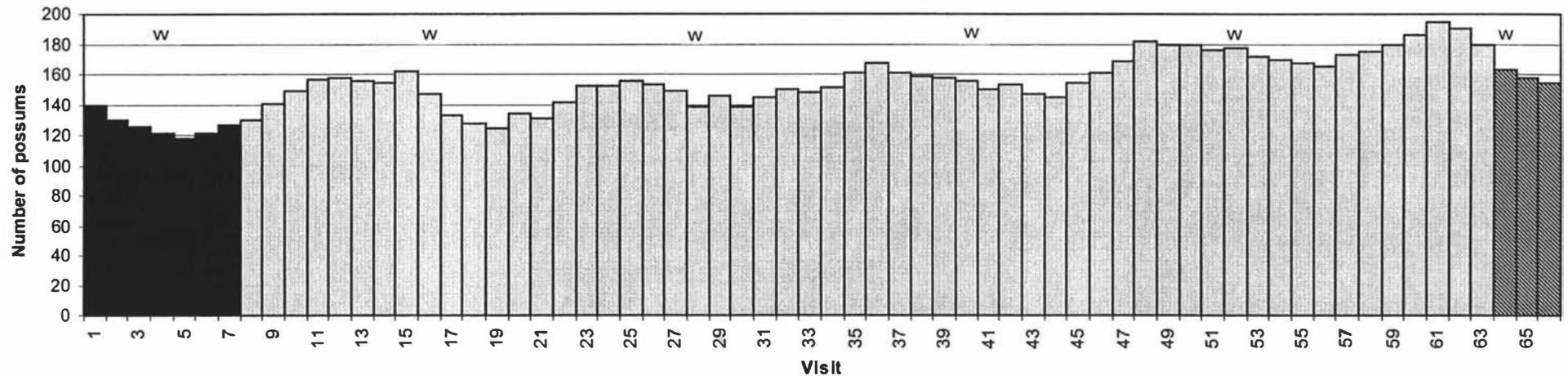


Figure A-2. Population estimate of possums on the Castlepoint study site. Black bars show where estimates have been manually extrapolated from later data trends. Hatched bars indicate period in which neighbour trapped and killed a number of study site possums. “W” denotes the mid-winter month of July

The population size for each month was estimated from Jolly-Seber capture-recapture analysis (Seber, 1982), using the software package Caro (S. Pledger and A. Tokeley, Victoria University of Wellington, and M. Efford, Landcare Research, Dunedin, 1995). Population estimates for the first 7 months were inaccurate and had very large standard errors due to problems inherent in commencing the longitudinal study (Pfeiffer, 1994) and the limitations of the method of estimation. These calculated values were discarded in favour of hand calculated estimates of the population taking account of the following months population estimates, and the predictable seasonal variation in possum abundance.

Appendix III. Estimation of possum condition index of the Castlepoint possums

The condition index of possums, a measure of fat/protein reserves, was calculated from the relationship of the body weight (kg) and the overall body length (cm). The data from 4995 measurement records on over 900 possums was used to develop a univariate linear regression model which investigated the relationship between the length (\log_e transformed) and the body weight (\log_e transformed). Similar procedures have previously been used by Bamford (1970) and Hickling *et al.*, (1991).

Length measurements

Repeated length measurements for each possum, which were recorded in the field, tended to vary by several cm, even when it was evident that the possum had ceased growing. This was attributed to individual operator error and variation in measurement technique. Possums measured when dead (post rigor mortis), also tended to be several cm longer than when alive. The crude field data for each mature possum with multiple examination records was adjusted manually in an attempt to correct this problem. Data for each possum was listed chronologically, and where it was evident that the possum had ceased growing, the total mature lengths were adjusted to a whole number mean value which was thought to best represent the overall length. These adjusted values were used in all analyses involving the length of possums in this thesis.

Regression analysis results

The results of regression analysis are shown in Table A-I.

Table A-I. Results of linear regression analysis examining the relationship between possum body weight (kg) and adjusted overall body length (cm), both \log_e transformed, taken from 4995 examination records.

Independent variable	Coefficient (95%CI)	T - value	P - value
intercept	-11.812 (-12.022 to -11.600)	-109.6	<0.001
\log_e length	2.920 (2.871 to 2.969)	116.7	<0.001

Adjusted $R^2 = 0.732$

The resulting equation used to calculate condition indices was:

$$\text{Condition Index} = 10^6 \times \frac{\text{Weight}(kg)}{\text{Length}(cm)^{2.9204}}$$

Where 10^6 is simply a multiplicative constant used to produce a variable with a scale from 0 to 15. The exponent for length was similar to that found by Fraser (1979) in a high density established possum population in the Copeland Valley. The data was also divided according to sex to investigate whether the gender had any influence on the condition indices. The two resultant regression lines were compared for both parallelism, and common intercepts (Kleinbaum *et al.*, 1988). Neither test showed significant differences between the sexes, thus validating the application of the equation above to possums of either sex. No sex differences in growth curves of possums were found by Bamford (1970).

Appendix IV. Estimating the age of possums examined in the Castlepoint study

Ages of 979 possums were estimated by six different methods depending upon what data was available for each animal, and the age at which first measurements on the possum were collected. The methods employed are listed below, ranging in order from the most to the least accurate.

The first method was based upon a regression equation derived from pouch young records of head length (mm) (see below). The second method was based upon possums being captured as independent animals of less than 2.2kg body weight, where they were still in a rapid growth phase, and age could be estimated from a predetermined regression equation (see below). The third method involved the estimation of year and season (Autumn or Spring) of birth in possums with an overall length which increased slightly after the first capture, and had teeth wear scores which suggested that the possums were young adults only. The fourth method was based upon sectioning molar teeth from dead animals, and counting the cementum annuli (Pekelharing, 1970; Clout, 1982). The fifth method estimated ages from the wear patterns of the upper premolar teeth only (Winter, 1980; Cowan and White, 1989). The sixth method involved having an educated guess at the age, because of the absence of tooth wear scores, or teeth for sectioning, in animals of mature body weight. The above procedures, commencing with pouch young head length, through to educated guessing, were used in 125, 479, 186, 22, 143 and 24 possums respectively.

The age estimates for pouch young were made after developing a regression equation which would allow prediction of age based upon the length of the head from tip of nose to occiput. The pouch young used to develop the equation had heads measured initially at 10 mm or less in length, and thus were less than 11 days of age according to the nomogram of Lyne and Verhagen (1957), which for these possums could be used to accurately estimate their date of birth. The regression data was derived from the 153 growth records from 56 pouch young of 43 different mothers. The results of the regression analysis are shown in Table A-II.

Table A-II. Results of linear regression analysis examining the relationship between age (days) and pouch young head length (mm) (n = 153).

Independent variable	Coefficient (95%CI)	T - value	P - value
intercept	-15.34 (-18.32 to -12.33)	-10.11	<0.001
head length	2.62 (2.54 to 2.71)	60.17	<0.001

Adjusted R² = 0.96

The predictive linear regression equation shown below:

$$\text{Age (days)} = -15.32 + 2.662 \text{ Head length (mm)}$$

Use of this predictive model resulted in age estimations very close to those of the nomogram of Lyne and Verhagen (1957). The model above was also similar to that developed by Bell (1981), shown below:

$$\text{Age (days)} = -20.914 + 2.915 \text{ Head length (mm)}$$

The difference in the two equations suggests that the possums of the Orongorongo Valley grow more slowly than those at the Castlepoint study site. The predictive value of the equation developed in this study is useful for possums up to a head length of 60 mm, but as head length increases the accuracy of the predictions falls substantially, such that at 60 mm the range in age of possums used in the regression modelling was from 108 to 182 days.

The regression equation for predicting the age of immature possums below a weight of 2.2 kg was developed from a data set of 123 possums whose ages were estimated accurately from pouch young head length records. From these 123 possums 504 data points were used in the regression analysis shown in Table III. Although both body weight² and overall length² were examined as independent variables it was found that there was no improvement in the predictive ability of the model based on body weight, through the incorporation of length data

Table III. Results of linear regression modelling, examining the relationship between \log_e age (days) and body weight² (n = 504).

Independent variable	Coefficient (95%CI)	T - value	P - value
intercept	5.237 (5.185 - 5.289)	198.0	<0.001
body weight ²	0.245 (0.227 - 0.264)	26.3	<0.001

Adjusted R² = 0.58

The predictive equation arising from the above model and which was used to determine the approximate age of 479 possums was:

$$\text{Log}_e \text{ Age (days)} = 5.2370 + 0.2453 \text{ Weight}^2$$