Transmission of tuberculosis (*Mycobacterium bovis*) by possums

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CHAPTER 3

Page 73, 3rd and 4th sentences should read: Efferent vessels from the tonsil pass directly to the deep cervical lymph node which is drained by the tracheal trunk. The mandibular and parotid lymph nodes drain independently to the superficial cervical lymph node.

Page 75, paragraph 4. Add: For those lymph node measurements not clearly defined in the text, average sizes were calculated from diameters measured at the widest point of the lymph node.

Page 78, last paragraph should read: *Efferent vessels.* On each side, a large vessel, the tracheal trunk, passed along the ventral surface of the longus colli muscle to a lymphaticovenous connection at the base of each external jugular vein.

CHAPTER 4.

Page 89, 2nd sentence should read: *Mycobacterium bovis* was not re-isolated from ribbons placed on pasture after 4 days.

Page 96. Add:

Survival on control ribbons

The results of cultures of a control ribbon kept in the laboratory at room temperature in the autumn and of four control ribbons kept at 5 °C are shown in Table 4.1a.

Table 4.1a. Culture test results from control ribbons maintained at room temperature or at 5 °C in the laboratory

	2 days	4 days	7 days	14 days	28 days
Control (room temp, autumn)	nt	tnc	tnc	67 cfu	-
Control 5 °C autumn	nt	tnc	tnc	tnc	tnc
Control 5 °C winter	nt	tnc	tnc	tnc	tnc
Control 5 °C spring	nt	tnc	tnc	tnc	tnc
Control 5 °C summer	nt	nt	tnc	tnc	+

nt = not tested; tnc = too numerous to count; - = *M. bovis* not cultured; + = *M. bovis* cultured; cfu = colony forming units

CHAPTER 5.

Page 115 paragraph 2. The reference, Tyndale-Biscoe (1955), applies to the statement in the first sentence.

Page 133, paragraph 2. The reference, Kent PT, Kubica GP. Public Health Mycobacteriology. A Guide for the Level III laboratory. US Department of Health Education and Welfare, Atlanta, USA 1985, applies to the statement about losses during decontamination in the 4th sentence.

CHAPTER 6.

Page 145, paragraph 2. The 4th sentence should read: During postmortem examinations in the studies detailed in Table 6.1, urine, faeces and tracheal washings were collected from randomly selected tuberculous possums with gross lesions of tuberculosis, from which tissues were also taken for bacteriological and/or histopathological examinations.

CHAPTER 8.

Page 204, 2nd last line: "trapping" should read "tracking".

Abstract

Tuberculosis caused by *Mycobacterium bovis* was diagnosed in 59 of 632 possums (*Trichosurus vulpecula*) individually identified over a 52 month period, during a longitudinal study of the naturally occurring disease in possums at a 21 hectare bush pasture location on a farm at Castlepoint in the Wairarapa. The disease exhibited marked spatial and temporal clustering and was continuously present in the population for the whole period.

The disease had a relatively long duration of up to 22 months and four distinct stages were demonstrated in cross-sectional studies. Among tuberculous possums, prevalences of up to 0.15 (±0.11) were recorded in the first stage prior to development of gross lesions. After dissemination started, the disease showed rapid generalisation to multiple sites by haematogenous and/or lymphatic spread to the next stage when gross lesions were evident, particularly in lung, axillary and inguinal lymphocentres. In the third stage, lesions were disseminated through almost all lung lobes, discharging fistulae were common and kidney, intestine and mammary gland were commonly affected by both gross and microscopic lesions. Behaviour and outward signs of health were unaffected prior to the terminally-ill stage, lasting for up to 2 months.

In common with other marsupials studied to date and in contrast with most eutherians, there are no popliteal lymph nodes and efferent drainage from the inguinal lymphocentre passes directly to the deep axillary group of lymph nodes via an inguinoaxillary trunk. All subcutaneous lymph drainage passes through either the superficial cervical or the axillary lymphocentres before entering the venous system.

Studies of survival of *Mycobacterium bovis* organisms in different natural habitats showed a relatively short period of survival of *M. bovis* outside hosts and support a conclusion that environmental contamination of pasture, particularly in summer months, may be relatively unimportant in the epidemiology of tuberculosis in cattle, deer and possums.

The weight of evidence favours transmission of infection by the respiratory route and it would seem that transmission of tuberculosis between possums occurs through two major and one minor pathway. The first major pathway is pseudo-vertical transmission from mother to joey during the rearing process. The second major transmission mechanism is direct horizontal transmission

among adult possums with available evidence suggesting that this takes place around the locality where a possum dens, probably during competition and threat/agonistic behaviour and during courting and mating activity. The third and probably least important pathway is indirect transmission among mature possums.

None of three ELISA assays reliably detected possums infected with tuberculosis and poor test performance was exacerbated by inconsistency between results from serially collected samples from known tuberculous possums.

Acknowledgements

I undertook this postgraduate training mainly to better equip me for solving problems in a logical and systematic manner, and to help me make sense of complex issues. I was very fortunate to be given an opportunity to work on a high profile project by my chief supervisor, Professor Roger Morris, who was willing to take on a "bush vet" who scarcely knew the difference between a mean and a median. I am very grateful to him, not just for that opportunity, but also for his generous assistance and responsiveness to needs throughout the study period and for his counsel through my transition from practice to research.

My other supervisors, Dr. Geoff de Lisle and Associate Professor Roger Marshall have constantly encouraged me and given of their time and expertise willingly and I am grateful to them both.

Life within the epidemiology group at Massey University under Professor Morris has been exciting and challenging and has given me a lot of fun and pleasure. All of the postgraduate students and staff in this group have been enthusiastic and loyal and supportive of one another. I thank them all very sincerely for their assistance at various times.

Within the group, Dr. Dirk Pfeiffer has guided me over and around many of the "brick walls" thrown up by analytical techniques and I have particularly enjoyed our many discussions about epidemiological issues.

The long term nature of longitudinal studies makes them more prone to problems than short term studies. We had our share of problems at Castlepoint, but none became serious, and a large part of the smooth running of the study was due to the good sense and friendly cooperation of my good friends Ron Goile, manager of Waio and his partner, Donna Lewis, both of whom have made an outstanding contribution to the longitudinal study. Thanks are also due to Bill Maunsell, the owner of Waio, who has made a quiet but considerable contribution by making his property available for the work.

The use of the library at Massey University has given me much pleasure over the past five years, but the greatest pleasure came from the human resource within the Faculty of Veterinary Science,

which shared its talents and knowledge willingly. Fiona Dickinson, secretary to Professor Morris, is a great exponent of all things pertaining to word processing and document layout, and she in particular has answered numerous requests for help and guidance in the preparation of this thesis and other reports.

The nature of my studies carried with it a high ethical cost in terms of use of animals. I trust that I used them wisely and was not wasteful in the extraction and use of information from them.

I am unable to adequately express my feelings for the most outstanding person of all. June and I have now been married for 37 years and her loyalty and support for me have been remarkable. It can't have been much fun helping me go round the trap lines on occasions in foul weather, or scribing for me in a cold shelter and then facing a waist deep evening return through Flagstaff Creek in mid-winter in Westland. Such is the mettle and loyalty of June, which she constantly has demonstrated throughout our life together. Our children too have been highly supportive of us in this venture and I am grateful to them for that. We miss our friends from Alexandra very much and thank them for continuing their friendship with us over time and distance.

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CHAPTER 1

Introduction

INTRODUCTION

In 1989, as part of a national integrated effort, a series of epidemiological studies under the direction of Professor R.S. Morris at Massey University, Palmerston North, was undertaken to define and clarify the role of the brushtail possum in the problem of tuberculosis in animals in New Zealand to enable that information to be used to design control methods which would reliably reduce the prevalence of tuberculosis in cattle and deer. At that time it was not known whether the possum was a true reservoir host, and its relationship with tuberculous feral pigs, ferrets and cats and their uncertain role in the maintenance of disease in domestic stock was regularly questioned by farmers, pest managers and scientists. At that time it was clear that possum pest control programmes were inadequate to eradicate tuberculosis in possums and there was a recognition that new and improved control programmes were required.

It was not known how cattle acquired the disease from possums. There were many theories but it was most commonly thought that cattle became infected through grazing pastures contaminated by tuberculous possums, particularly at the forest pasture margins, where prevalence of tuberculosis in possums was thought to be highest. No longitudinal studies had been made and there was consequently no knowledge of the temporal patterns of the disease in possums. The course of the disease was thought to be rapid and the host response poor and inadequate. Experimental studies had been carried out but knowledge of the naturally occurring disease relied on case studies which had used either non-random case selection and/or non-standardised necropsy procedures. The interpretation of pathological findings suffered because the gross anatomy of the lymphocentres and lymphatic pathways had not been described.

Within the general scientific community there was a mediocre understanding of tuberculosis, many features of which had been established by researchers during the first half of the century. As was the case in human medicine, measures which had given acceptable initial progress in tuberculosis control had led to a sense of complacency, although in the absence of problems, there had been no real requirement to have a high level of understanding.

The Massey University studies were part of an integrated national and international scientific effort to formulate short, medium and long term control programmes. The series of studies described here were a natural progression from the longitudinal study at Castlepoint designed by Pfeiffer (1994), and a part of this thesis describes the continuation of that longitudinal study.

Studies of the many complex issues of tuberculosis have seldom produced clear-cut conclusions and have commonly led to controversy. Wildlife disease has been described by Morris (1995) as the ultimate epidemiological challenge, largely because of the problems of estimating population at risk and calculating the usual indices used to characterise the epidemiology of a disease. Thus the study of tuberculosis in a wild species presents a particularly daunting challenge, not only from the inherent difficulties associated with tuberculosis and dealing with uncontrolled wild animal populations, but also from acceptance from the general and scientific community that many conclusions will necessarily be derived by inference from multiple sources and from consideration of established general principles of host response to infection with *Mycobacterium bovis*. Where possible within this thesis, conclusions and inferences have been drawn following assessment using epidemiological techniques which tested the goodness of fit of particular explanations, and there has been a strong underlying endeavour throughout to make deductions which have application to control programmes.

CHAPTER 2

Tuberculosis caused by *Mycobacterium bovis* in New Zealand

PROGRESS AND CURRENT STATUS

Bovine tuberculosis was introduced into New Zealand by tuberculous cattle at the time of European settlement but the first national scheme for control did not start until 1945. This scheme was prompted by public health concern about the sale of milk from tuberculous cattle in town supply herds and was accompanied by legislation which directed that test positive cattle be slaughtered. The scheme was voluntary at its start but progressed to compulsory participation with all town supply herds under test by the end of the 1950s. In 1961, the scheme was extended to the manufacturing dairy industry which supplies milk for the export trade in dairy products. Beef cattle testing on a national basis started in 1967, and by 1977, all breeding cattle in New Zealand were under test and a surveillance system was in place at slaughter houses for all beef animals.

The dairy factory supply and beef cattle control schemes were undertaken primarily with the aim of ensuring continued access to competitive international markets but it is now recognised that changes in consumer perceptions about the wholesomeness and safety of New Zealand meat have the potential to pose even greater threats to profitable marketing. The total trade at risk is:

Dairy	$3.42 billion	
Beef	$1.10 billion	
Venison	$0.12 billion	
Velvet	$0.06 billion	
Total	$4.70 billion	source Livingstone (1995)

Initial progress in controlling the disease was rapid, although in some areas prevalence was very high, with up to 60% of herds infected. By 1979-80, the percentage reactors had reduced from an initial 8.6% to 0.05% in dairy cattle and from 1.5% to 0.1% in beef cattle. Since that time, there has been little positive progress in the reduction of incidence of reactors. The continued effort has only held reactor incidence steady and has had an undetermined effect of reducing the rate of dissemination and expansion of endemic areas. The reason for the continued lack of progress is the widespread established Australian brushtail tuberculous possum (*Trichosurus vulpecula*) populations and the reservoir status of possums for the disease. The New Zealand population size was estimated to be approximately 70,000,000 by Batcheler and Cowan (1988).

For reasons of control, Ministry of Agriculture and Fisheries (MAF) authorities have categorised New Zealand into either Special Tuberculosis Control Areas, (STCAs), in which tuberculosis is endemic, or Surveillance Areas, where the disease is not endemic. An STCA has a central endemic zone, surrounded by a "fringe" zone, which is in turn surrounded by a non-endemic zone. Each zone is defined geographically and takes security afforded by local terrain into account. The central endemic zone comprises a region containing known tuberculous possums, the fringe zone covers an area considered wide enough to contain tuberculous possums which might migrate into it, while the surrounding non-endemic zone provides further confidence of containment. An area is declared endemic when tuberculosis is found in wild-life within that area. The non-endemic areas contain minor endemic areas which are well defined geographically and these areas are termed Special Tuberculosis Investigation Areas (STIAs) (Livingstone, 1992). It is conceded that the disease cannot be eliminated from STCAs at this stage but eradication of disease from STIAs is considered technically feasible and is currently underway in some of these areas.

The number of endemic areas increased from 9 in 1980 to 23 at present, (6 STCAs and 17 STIAs), but more importantly, the area of New Zealand which is classified as endemic increased from 10% in 1980 to about 24% at present and contains approximately 12% of all herds. The endemic areas as at 1992 are shown in Figure 2.1 (reproduced from Livingstone, 1992) and the STCAs as at 1995 in Figure 2.2 (reproduced from Animal Health Board information sheet).

For the most part, cattle and farmed deer contract tuberculosis after direct contact with tuberculous farmed animals or wildlife reservoirs. Of the non-farmed species, the possum is currently considered to be by far the most important. The slow progress in reducing the incidence of disease is evident in Table 2.1 (compiled from Livingstone, 1992, and Chief Veterinary Officer, 1992; 1994).

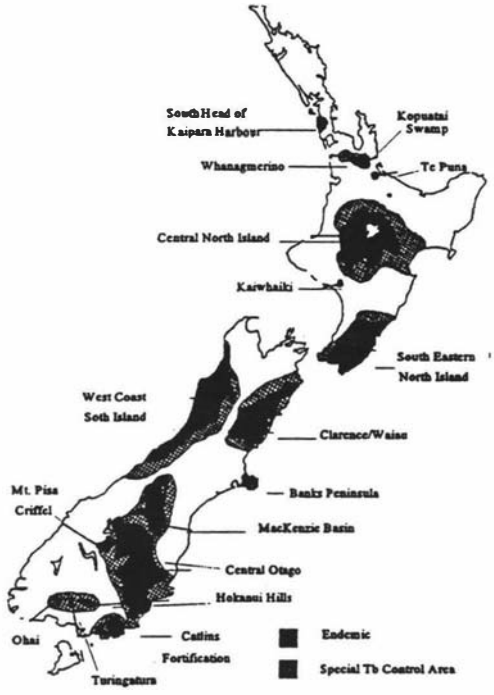

Figure 2.1. Map of New Zealand showing areas endemic for Tb and Special Tuberculosis Control Areas (September 1991) courtesy P. Livingstone

Figure 2.2. Areas of Endemic Tb (shaded black) in New Zealand 1995

Table 2.1. Number of cattle reactors , lesion-non-tested cattle, and movement control (MC herds with cattle reactor incidence rates (%) for STCAs and Surveillance Areas of New Zealand for the testing seasons 1985/6 to 1992/93.

Summary data								
	85/86	86/87	87/88	88/89	89/90	90/91	91/92	92/93
No of reactors	data not reported in same format as in later years				5768	5922	5940	6183
Lesion-non-tested					712	871	689	831
No of M C Herds					1231	1292	1314	1383
Reactor incidence %								
STCAs	0.432	0.386	0.418	0.424	0.357	n.a.	n.a.	n.a.
Surveillance	0.048	0.044	0.074	0.031	0.069	n.a.	n.a.	n.a.
New Zealand	0.232	0.208	0.239	0.222	0.239	0.21	0.20	0.18

n.a. = not available

Lesion-non-tested refer to animals detected at slaughter. These animals are not included in the calculation of reactor incidence.

$$Reactor\ incidence = \frac{No.\ of\ tuberculin\ reactors\ slaughtered}{No.\ of\ cattle\ under\ test}$$

Reactor incidence is the best available measure of the rate at which cattle are becoming infected. Calculation of a true rate is difficult because not all cattle are tested each year and some herds are tested more frequently than once annually.

Testing regimes

The basic testing regimes differ between STCAs and Surveillance areas. In STCAs, all stock older than 6 to 12 months are tested annually with the caudal fold intradermal tuberculin test and for test interpretation any visible or palpable injection site swelling is classed as a reactor. In Surveillance areas, breeding stock from 12 to 24 months of age are tested every 2 to 3 years and only injection site swellings which are greater than 4 mm are considered reactors. In large herds in Surveillance areas, a sample of up to 250 animals is considered sufficient to determine herd status. Accredited deer herds in Surveillance areas are tested every two years. Cattle in endemic areas or from movement control herds are tested prior to and after movement to new areas.

When reactors are detected, the frequency of testing is increased. The time periods for the steps in moving from infected herd status to free status are subject to change, but generally follow the following pattern: Initially an infected herd is classed as MCI (Movement Control Infected). If the following test is clear the herd is classed as MCC (Movement Control Clear). One more clear test gives F (Free) status. Two F tests are required before A (Accreditation) status.

The present effort is maintaining the status quo as far as reactor incidence rates are concerned, is almost certainly having some suppressive effect on the rate of extensions to existing endemic areas and is inhibiting creation of new endemic areas. However, the real concern is that the total area of endemic areas is increasing and has increased steadily over the past 20 years, although part of the increase in size may have been due to better definition of previously poorly defined areas.

Both herds and individual cattle are at a higher risk of exposure to *M. bovis* in endemic areas than in non-endemic surveillance areas. The probability of a herd becoming infected in endemic, fringe and STIAs is about 16, six and eight times respectively greater than the risk for becoming

infected in surveillance areas. Eight-eight percent of slaughtered reactor cattle and 93% of MC herds came from STCAs in 1989/90 (Livingstone, 1992), indicating that endemic areas were well defined geographically.

For an area to be classed as endemic, tuberculosis must be confirmed in wildlife, but detection of disease in possums or other wildlife relies on time-consuming and expensive surveys involving killing and necropsy. The efficacy and reliability of this method of detection has not been determined and there is always concern that negative results may be false. A large number of possums need to be examined in surveys to detect disease if prevalence of gross lesions at autopsy is low. If the prevalence is 1% and diseased animals are randomly distributed, approximately 300 possums need to be examined to be 95% certain of detecting one diseased animal from a large population. A larger sample is required for examination if the disease occurs in clusters of infected animals, a situation which applies to tuberculosis in possums (Coleman, 1988; Hickling, 1991; Pfeiffer, 1994).

The status of Surveillance areas is at risk from two possible methods of spread of infection into possum populations.

The first possibility involves spread from existing MC herds, untested tuberculous herds or tuberculous herds which have not been recently tested and have accredited status Past experience of dealing with tuberculosis in cattle herds suggests that the risk of spread from the above sources is very low. There is general agreement that the transmission of infection from cattle to possums is a rare occurrence. Maintenance of the prevalence of disease at a low level through regular testing with slaughter of reactors reduces the probability of such an occurrence even further (Morris *et al.*, 1994).

The present level of testing appears adequate to detect herd infection and natural control is probably assisted by husbandry and ecological factors within the surveillance area which act as natural barriers to disease establishment. This combination of a low probability for transmission from cattle to possums, early detection systems for disease in cattle and existing natural ecological barriers reduces the risk of areas becoming endemic.

When an unusually high prevalence of disease is detected in a herd, attention is directed to neighbouring herds and the animal population within that area, for early containment and resolution of the problem.

Deer herds with high prevalence of tuberculosis are known to have established endemic disease in some locations and such herds are given special attention. Following detection of high risk herds, an early decision is usually made as to whether total depopulation of the affected herd is warranted along with vigorous possum control. A compulsory testing and slaughter policy for deer was introduced about 1989 and the likelihood of transmission of tuberculosis from farmed deer to possums should now be very low.

The more likely and more serious method of spread involves migration of tuberculous possums from endemic areas. This poses the more serious risk because of difficulty of detection at an early stage and difficulty for containment. If a disease outbreak in farmed animals comes from introduced diseased farm animals, it is normally detected at a relatively early stage during routine surveillance testing. If on the other hand, possum dispersal from an endemic area introduces disease into local possum populations, detection may be delayed, and meanwhile the infected possum population may expand further. Infected possums probably need to come into close contact with susceptible farm animals for successful direct transmission and such contact probably occurs relatively uncommonly (Paterson, 1993).

During a lag phase, infected dispersing possums have opportunities to establish new foci of infection at the limits of their dispersal ranges. A further delay in detection in local cattle at new foci means that an infected possum front may be well in advance of recognised disease in cattle.

Containment of infected possums is achieved by creating poison buffer zones (fringe areas). The widths of buffer zones are set sufficiently wide to contain dispersing animals. Livingstone (1988) estimates that the average yearly spread over a 7 year period in the vicinity of Mangakino, into the Tirohanga dairying area, and through the pine plantations into the Tutakau Road area, was approximately 5 km per year.

Animal Health Board strategic plan

A strategic plan for 1993 to 1998 dated October 1992, was drafted by the Animal Health Board (AHB). It proposed intensification of possum control in and around endemic areas and set out the following objectives:

(a) In endemic areas to reduce the percentage of MC herds (deer and cattle combined) by 30 - 50% and the number of reactors by 50 - 70%.

(b) To reduce the percentage of MC herds in the non-endemic areas to 0.2% (the internationally recognised level for official freedom from tuberculosis).

(c) To prevent the establishment of new endemic areas and expansion of existing endemic areas into farmland free of feral/wild vectors.

(d) To encourage individual farmers to take responsibility for the control of tuberculosis within their herds.

Only one non-endemic region, viz. Bay of Plenty, had achieved the 0.2% status at January 1992. In the Central North Island endemic area, 21.7% of cattle herds and 27.2% of deer herds were on movement control at January 1992 compared with 1.95% of cattle herds and 2% of deer herds in the non-endemic regions.

Control costs and funding

The possum has an enhanced pest status because of its role as a reservoir and vector of bovine tuberculosis and deciding the best way to deal with this pest presents a real dilemma to control agencies. Livingstone (1991) estimated that approximately $700 million would be needed to be spent over the next 20 years using current control techniques to eradicate tuberculosis from possums. He also conceded that this was not a viable option on the grounds of cost, the uncertainty of being able to achieve a successful outcome and heightening public concern over the use of 1080 poison.

The Animal Health Board budget for 1994-95 proposed an expenditure of $36.1 million, estimated to rise to $43.4 million for 2000/01, allocated as follows (Animal Health Board, 1995):

Expenditure	1994/95	2000/01
Reactor compensation	1.8	1.8
Testing/epidemiology	9.3	9.3
Scheme management	4.6	4.9
Research*	1.8	2.0
Information/education	1.1	2.1
Vector control	17.5	23.3

TOTAL (millions)	$36.1	$43.4

* AHB funded research only. Total funding for tuberculosis related research is $12 million per annum, of which 75% is funded by the Crown.

Funding for 1994/95 will be derived from:

Industry levies, grants and sponsorships	21.5
Landowners/regions	3.5
Government	9.8
Industry (direct payments)	11.9
TOTAL (millions)	$46.7

Farmer monetary input into possum control through a levy on all cattle slaughtered is substantial and has increased dramatically over the past few years. The Animal Health Board has taken responsibility for co-ordinating the control effort by control of spending and policy making, although until recently it lacked full legal status. The Biosecurity Act 1994 gave management powers to the AHB to enable it to levy funds, develop and implement strategies for the eradication of *M. bovis* tuberculosis. The Biosecurity Act requires a consultative approach to pest (includes *M. bovis*) management be followed, directs responsibility for control programmes to those involved, and requires that the benefits of implementing the management strategy must outweigh the costs. Currently the Animal Health Board is conducting a consultative process to help it decide the best mix of individual and centralised responsibility for tuberculosis control. The Board has conceded that a long term research effort and prolonged control will be necessary to solve the problem of bovine tuberculosis (Alspach 1991).

Some appreciation of the daunting nature of possum control in New Zealand and the limitations of the methods used can be gained on reflection that despite the vigorous control measures which have been in place for the past 40 years, the possum population has continued to increase and colonisation of new areas has continued.

THE CASE FOR TUBERCULOUS POSSUMS BEING A SOURCE OF TUBERCULOSIS FOR CATTLE

Tuberculosis was first recorded in possums caught by a trapper on a farm at the mouth of the Mokihinui River, Buller County, in September 1967 (Ekdahl *et al.*, 1970). Twenty of the 25 possums which he submitted for examination showed gross lesions of tuberculosis. Attention was further focused on the possible role of the possum when, in 1970, there was a serious outbreak of tuberculosis in a dairy herd at Seddonville, just a few miles upstream from the original discovery of tuberculous possums.

The area in which this outbreak occurred supported a large possum population and was bounded by bush on three sides and by the river on the fourth. A post-mortem survey of possums in the vicinity revealed 12% prevalence of the disease. The isolation of these cattle by physical boundaries from other cattle and the presence of a significant level of disease in possums suggested an hypothesis that possums were acting as vectors of the disease. Up to this time, it had been considered that cattle contracted the disease from tuberculous in-contact cattle.

On the basis of these findings it was decided to further investigate the role of the possums as a source of infection. The area in which the outbreak occurred was deliberately destocked for a period of six months. Twenty-nine tuberculin tested negative calves were then introduced to graze the area. Six months later, 26 of these animals reacted to the caudal fold intradermal test (CFT) and of these, 16 exhibited gross lesions of tuberculosis at slaughter. (Davidson, 1976). This was the first real evidence which implicated possums as a source of infection.

Although the disease in possums warranted prolonged and intensive investigation at this early stage, only limited studies were in fact carried out. Consequently, for many years control of the disease was hampered by significant deficiencies in the understanding of tuberculosis in possums. More recently, comprehensive and detailed studies have been undertaken by researchers at Massey University, Wallaceville Animal Research Centre, Landcare and within the Ministry of Agriculture and Fisheries (MAF) to remedy those deficiencies.

Ekdahl *et al.*(1970) and Smith (1972) have reported on the nature of the disease in possums and Smith drew attention in particular to the large numbers of *M. bovis* organisms which were present in lesions in affected animals. Some experimental infection studies were made by O'Hara *et*

al.(1976) in New Zealand and Corner and Presidente (1981) in Australia, which indicated rapid progression of disease and a presumably limited host response to counteract the infection, leading to the presence of large numbers of organisms in lesions. In summary, these early studies showed the possum to be a susceptible host with a capability of producing large numbers of *M. bovis* organisms, thus having the potential to be a potent vector of the disease.

There has been a gradual accumulation of evidence during the past 25 years incriminating the possum as the most important source of *M. bovis* infections in cattle. Indictment of the possum comes not from a single source but from collective evidence from multiple studies.

Evidence from tuberculin testing

Some evidence of the role of possums in maintaining tuberculosis in cattle comes from the results of routine testing and the inability of a test and slaughter policy to eradicate the disease in certain parts of New Zealand. The intra-dermal tuberculin test has been the basis of the tuberculosis eradication scheme which was instituted in New Zealand in the 1950s This test is reliable, it has been used world wide and its use in national control programmes has enabled eradication in most countries where the source of infection has been confined to cattle.

The situation faced on the West Coast of the South Island in the early 1970s had presented a new problem to the Ministry of Agriculture and Fisheries. Close examination of testing records for the region revealed unexpected difficulties being experienced in the testing program which could only be explained by the presence of a source of herd re-infection other than cattle. Conversely, and in line with predictions and expectations, many areas of New Zealand were experiencing no difficulties with eradication.

Unfortunately the problem of herd breakdowns was not long confined to the West Coast region. Other regions followed. The northern region of the West Coast was the next area to report tuberculosis in possums, while in 1969, the Wairarapa also identified possums as a factor in intractable herd problems. The Western Bays of Lake Taupo, Central North Island followed in 1972 (Batcheler and Cowan, 1988). The situation continued to deteriorate after the primary recognition of the particular problems in these three regions. Other areas in which the disease was initially considered eradicated from cattle have subsequently joined the list of endemic areas. In 1991, there were 19 defined areas wherein tuberculous possums have been found (Livingstone,

1991). Since that report, two additional endemic areas were notified, at Waipawa in Hawkes Bay and Otaki in the Manawatu, while at least two other areas were placed under investigation.

As previously pointed out, cattle in STCAs are more likely to be slaughtered as Tb reactors than cattle in surveillance areas (Livingstone, 1991; Chief Veterinary Officer, 1994). In other words, the risk of cattle becoming infected is much higher in those areas where the presence of tuberculous possums has been confirmed.

Tuberculin testing evidence thus incriminates the possum on two grounds, viz. the inability of a proven test to eradicate the disease and the higher risk of cattle becoming infected in areas containing tuberculous possums.

The incidence of cattle tuberculosis following possum poisoning operations

The hypothesis that tuberculous possums are responsible for cattle tuberculosis has been tested by monitoring the course of events following removal of tuberculous possums by control operations.

The incidence of tuberculosis, i.e. the rate at which new cases appear, reduced after possum poisoning operations. This observation has been made a number of times in different locations (Pannett, 1991; Hoyle, 1991; Anon, 1986).

Restriction endonuclease typing of M. bovis

Subtle differences in the genetic makeup of *M. bovis* organisms are uniquely expressed in DNA sequences within genes. Scientists at Wallaceville (Collins and de Lisle, 1984; 1985) developed a technique based on restriction endonuclease analysis of DNA (Rea typing), which enables them to classify *M. bovis* bacteria into distinct types based on restriction fragment patterns. To date, they have identified in excess of 50 different patterns using this technique (de Lisle, pers. comm.).

Different restriction types were found in the three main endemic areas, viz. the Wairarapa, the Central North Island and the West Coast, indicating that problems in these areas evolved independently. Specific types have come from both farmed and non-farmed animals in the same localities (Collins *et al.*, 1986; de Lisle *et al.*, 1990). The types of *M. bovis* found in possums have been found to be the same as those found in cattle and deer in the same locality. This is consistent

with a cycle of infection between those species and adds further weight to the possum vector theory.

Persistence of tuberculosis in possum populations

For some time it was questioned whether tuberculosis is self- sustaining in possum populations or whether infected cattle are needed to maintain the disease by re-infecting possums. It is now clear that possums living in the ecological circumstances applying in New Zealand, where they occur in great numbers, have attained reservoir status (Morris *et al.*, 1994)

A longitudinal study of naturally occurring tuberculosis in possums in the wild at Castlepoint (Pfeiffer 1994) produced strong evidence to support transmission of infection from mother to offspring and also indicated that transmission between adults was largely confined to local social groupings, covering as little as 2 to 4 ha of den-sites. These findings strongly suggested that spread of disease may be related to behavioural factors associated with breeding activity, and possibly also with tree marking or concurrent or sequential usage of dens, although concurrent den-sharing appeared to be a rare event at Castlepoint. These latter findings point to transmission pathways capable of maintaining the disease in possums independently of cattle.

Analogy with badgers in the U.K. and Eire

Some regions of England and Ireland experience a problem with intractable tuberculosis in cattle which is very similar to the New Zealand problem (Nolan and Wilesmith, 1994). It was realised that a problem existed towards the end of the 1960s, and in 1971 an infected badger was found on a farm in Gloucestershire which had recently experienced an episode of tuberculosis (Muirhead *et al.*, 1974). Following these findings, a strong web of circumstantial and research evidence was gathered, similar to that presented here, which implicated the badger as a wildlife reservoir of infection. The most conclusive evidence came after two problem areas were depopulated of badgers. This step proved to be successful in preventing re-infection of cattle (MAFF, 1979; Little *et al.*, 1982). The existence of this proven wildlife reservoir in analogous circumstances to brushtail possums adds plausibility to the possum reservoir theory.

Why possums?

Tuberculosis caused by *M. bovis* occurs in an exceptionally wide variety of warm blooded animals (Francis, 1958; Lepper and Corner, 1983; Thoen *et al.*, 1984; Pritchard, 1988) but none of these species which occur in New Zealand is as abundant as possums, nor does any show the consistent

overall prevalences of tuberculosis which are found in possums. No other species presents a similar threat in terms of numbers of infected animals capable of transmitting the infection.

Summary

In summary, the evidence implicating the possum relies on circumstantial evidence from a variety of sources, viz.

(a) the disease occurs in cattle in contact with tuberculous possums

(b) possums are susceptible hosts capable of producing large numbers of organisms, qualities which are consistent with the concept of their being a successful reservoir

(c) cattle in STCAs are at a higher risk than cattle in surveillance areas

(d) tuberculosis in possum populations is self-sustaining

(e) analogy with badgers

(f) weight of numbers of infected possums, allowing many transmission opportunities.

It may be argued that this evidence is not fully conclusive despite its compelling nature. The argument would be strengthened if the tuberculosis status of possums in non-endemic areas was known and if total removal of infected possums from a region would prevent the recurrence of the disease in cattle. There would be considerable difficulties in obtaining comprehensive evidence regarding the tuberculosis status of possums in non-endemic areas and such an exercise would be an expensive undertaking. The sequence of events following eradication of tuberculosis in possums in "island" situations is presently being determined (Hoyle, 1991; Livingstone, 1991).

THE ROLE OF POSSUMS IN THE EPIDEMIOLOGY OF TUBERCULOSIS IN DEER

Although the reservoir and vector status of possums is well established, the same cannot be said with the same degree of certainty for deer, although it appears highly likely. The part that possums play in the epidemiology of the disease in farmed and wild deer has not been investigated to the same extent as in cattle.

The first case of tuberculosis in red deer was recorded in 1955 (Davidson, 1976), and interest was heightened when a case in a free living wild deer was reported from Mohikinui on the West Coast in 1970, an area where tuberculous possums were known to exist. Although there was a thriving wild venison export industry in the 1970s, the inspection of carcasses in the formative years was superficial and was not designed for the detection of disease. Consequently, there was no satisfactory determination of prevalence in wild deer made at that time.

In 1978, the first case of tuberculosis in a farmed deer was recorded on a property contiguous to forest containing tuberculous possums. The farmed deer on this property had originated from the adjoining forest (Livingstone, 1980, cited by Beatson 1985).

When improved inspection procedures (which included an inspection of viscera and the carcass of wild deer), were introduced through regulations in 1975, the number of notifications of tuberculosis increased. Between 1970 and 1983, 161 isolates of *M. bovis* were made from wild deer while a further 340 came from farmed deer (de Lisle, 1985). Most of these cases were recorded after 1978 and it is likely that the number of isolates from wild deer is an under-representation. This is because inspection, until recently, did not include the retro-pharyngeal lymph nodes which are the most commonly affected lymph nodes in deer (Livingstone, 1980; Beatson and Hutton, 1981; Wilcockson, 1986; Leeming, 1991). Despite this deficiency, the results clearly show that tuberculosis existed in free living wild deer in certain regions throughout the country. Infected wild deer have originated from the West Coast of the South Island, Southland, Ekatahuna County, the Wairarapa and the Central North Island. The disease has been found in wild red, fallow and sika deer (de Lisle and Havill, 1985).

Widespread trading and movement of deer accompanied by a voluntary tuberculosis testing programme created difficulties for determination of the source of infection for farmed deer.

Carter (1991) calculated a very high relative risk of 21 for the probability of deer herds in STCAs going on to movement control compared with herds in surveillance areas. However, the disease in farmed deer may conceivably originate from infected cattle, infected deer, infected premises or infected possums. The epidemiology should become clearer as infection in farmed animals on individual properties is better controlled and the confounding influence of movement between herds reduced. Control of tuberculosis in farmed deer has progressed satisfactorily.

The epidemiology of tuberculosis in farmed and wild deer is poorly understood but it is reasonable to assume that tuberculous possums may be responsible for infecting deer in the wild and on occasions, farmed deer. It is worthy of note and a matter of some concern that there is some evidence from investigations of disease outbreaks in Southland and the MacKenzie basin which incriminates tuberculous farmed deer as a source of infection for possums (Carter 1988).

THE EPIDEMIOLOGY OF TUBERCULOSIS IN POSSUMS

Introduction

Bovine tuberculosis was introduced into Australia and New Zealand by tuberculous cattle at the time of European settlement. Although the disease has persisted in cattle in both countries, there is no evidence to suggest that tuberculosis has ever established in wild possum populations in Australia, a situation which contrasts with the New Zealand experience. Australian veterinary authorities have recently reported that they have completed eradication of bovine tuberculosis from cattle herds throughout Australia. Many of the Australian herds share habitat with possums, but possums have never been implicated as a source of re-infection of cattle in that country, either during or following their successful eradication programme.

It is not known for how long the disease was present in possums in New Zealand prior to its initial recognition in September, 1967. This diagnosis was made at Wallaceville Animal Research Centre from a possum trapped at the mouth of the Mohikinui River. Tuberculosis had been present for some time on the farm where the tuberculous possums were first discovered and this same farm had experienced a very large increase in incidence of the disease in cattle on the farm about 4 months prior to the diagnosis of tuberculosis in possums.

In the neighbourhood over the next two years, the incidence increased in low incidence herds and new cases occurred in herds previously clear of the disease, suggesting that the disease had become established in possums not long before 1967, and had then spread relatively slowly among the local population in the lower Mohikinui valley.

On the other hand, there were farmer reports of a disease resembling tuberculosis having been discovered in the Buller and Inangahua area in the mid 1950s. In addition, tuberculous possums were found at widely scattered locations throughout the lower Buller County from late 1968 onwards, and it was considered unlikely that disease could have spread from Mohikinui with such rapidity (Davidson, pers. comm.).

In the North Island, tuberculosis was first found in a possum on a station in the Wairarapa in 1969, but the disease had probably been established in possum populations for some time prior to that event. A persistent cattle tuberculosis problem in the Wainui-o-mata valley near Wellington in the mid-1960s had been attributed to infected wild pigs, but in hindsight may have

originated from tuberculous possums. Infected possums were subsequently discovered in that location in 1975 (Batcheler and Cowan, 1988).

After initial recognition of the disease in possums, infected possums were reported in many widely separated locations throughout both Islands between 1970 and 1975 and it is reasonable to conclude that the disease had been well established in various possum populations for a considerable time before these discoveries. The investigation of the disease status of possums was prompted by increased awareness of the problem, and by the failure of regular tuberculin testing and slaughter of reactors to control the disease in cattle herds in some areas.

Disease investigations

Prior to the recognition of tuberculosis in possums in Westland County (Eckdahl *et al.*, 1970) and the suspected role of possums as a source of infection, the disease in possums had in fact been recorded on several separate occasions.

The first two cases of tuberculosis recorded in possums were in captive animals in zoological gardens (Moore, 1903; Scott, 1928). Before the advent of pasteurisation, improved meat sanitary codes and the adoption of better and more natural housing, zoo animals were particularly susceptible to tuberculosis, and the disease was relatively common among resident zoo animals.

During an Australian study of the susceptibility of marsupials to infection by pathogens of eutherian mammals, Bolliger and Bolliger (1948) successfully infected possums with *M. bovis* by the oral route and by intra-peritoneal and intramuscular injections. In this study they also reported transmission of infection from a tuberculous possum to an in-contact possum.

The disease in possums has since been studied using experimental infection techniques in laboratories, and in cross-sectional and longitudinal epidemiological studies of the disease as it occurs in nature.

Experimental infection studies

Experimental infection studies into the pathogenesis of the disease were first carried out in New Zealand at Wallaceville Animal Research Centre (O'Hara *et al.*, 1976). In this study, inoculation by the subcutaneous and intra-nasal routes led to successful infection which

progressed rapidly to fatal generalised disease. Direct and indirect transmission of disease between adult possums and transmission from an infected female to her pouch-young were also recorded.

As the scale of the New Zealand problem became clear in the 1970s, concern by the Australian veterinary authorities about the possibility of possum involvement with bovine tuberculosis led to further experimental infection studies (Comer and Presidente, 1980; 1981) in Australia, where a national bovine tuberculosis eradication programme was underway. The first study showed that intramuscular inoculation of *M. bovis* produced a bacteraemia after two weeks followed by the development of disseminated and generalised lesions after a further 4 weeks. Animals with generalised disease excreted large numbers of organisms via open sinuses and in faeces and urine. Similar findings were recorded in the second study, including successful transmission (presumed to be by aerosol) from an infected to a susceptible possum.

Similar pathological findings have been recorded by Buddle *et al.* (1994) and Pfeffer *et al.* (1994) in experimental infections in possums conducted as part of detailed immunological studies.

Rapid progression of the disease was a feature of experimental infection in captive animals, presumably as a result of the relatively large dose of infective organisms used to initiate infection, compounded by a reduction in natural immunity due to stress effects of capture and confinement (Buddle *et al.*, 1992).

In experimentally infected possums the disease was progressive and invasive with the production of large tuberculous nodules, often with central necrotic areas, and all of these studies have demonstrated that the possum is highly susceptible to the effects of experimental infection by *M. bovis*. Experimental infection studies generally produce valuable information and increase understanding of the disease process, but they often inadequately explain the way the disease behaves in nature.

Cross-sectional studies

A number of cross-sectional surveys were carried out in response to recognition of tuberculosis as a disease entity in possums in the wild. In this type of study, conducted for the most part to determine prevalence, possum populations were sampled by poisoning or trapping and the catch

necropsied. Many such surveys were carried out in various parts of the country by Ministry of Agriculture and Fisheries (MAF) staff. They have been particularly useful for demonstrating an association between the presence of tuberculous possums and tuberculous cattle and also have given some indication of the distribution of lesions in diseased animals.

More rigorously designed investigations to determine prevalence of tuberculosis in possums started in the early 1970s. In surveys in the Buller County from 1970 to 1971 inclusive, 202 diseased animals (4.8%) were found out of a total of 4193 animals examined. Eighty-nine diseased animals (5.2%) were found out of 1715 examined in Inangahua County (Cook, 1975).

This report records prevalences varying between 2.8 and 6% in various surveys carried out in Buller County between 1970 and 1974. Prevalences in similar surveys in Inangahua County varied between 3.4 and 7.8%.

Data from a survey carried out in 1973-74 in the Hohonu Range in Westland was analyzed by Coleman (1988) and he drew attention to several important epidemiological features of the disease in possums. An earlier study (Coleman *et al.*, 1980) had shown that population density in a locality was highest at the forest-pasture margin, and lowest at locations the most distant from pasture. A similar gradation of density as indicated by trapping was seen in the Hohonu study, plus an observation that prevalence of disease was highest in animals trapped on pasture. He also noted that tuberculous animals seemed to be distributed in disease foci of three to five animals, indicating that close direct or indirect contact might be involved in the transmission of disease. The highest prevalences of disease were recorded in autumn and winter at pasture and in spring within the forest.

Hickling (1991) summarised previously recorded data showing prevalences of 1.3% in the Hauhungaroa in 1983 (N = 6083), 2.3% in the Hohotaka in 1988 (N = 830) and 2.9% in the Hohonu in 1990 (N = 677). Hickling also noted patchy occurrence of the disease with average prevalences of 9% in foci of infection and a maximum level of 17%. An interesting observation (Hickling unpublished data) was that a comparison of the distributions of tuberculosis in possums in the Hohonu Range in a 1973 survey (Coleman, 1988) and in a 1990 survey showed foci of infected possums at the same locations, even after 17 years.

Coleman *et al.* (1994) reported 60% prevalence in a low density possum population at Flagstaff Flat in 1992 and further surveys in that area recorded prevalences of 17% in 1993 and 9% in 1994 (Coleman and Cooke, 1995).

These cross-sectional studies have shown a common pattern regarding the prevalence of disease in adults and immatures. In all areas studied, the disease appears to affect similar proportions of adult males and females (except for the Flagstaff Flat study of 1992), affect immature males more than immature females and have highest prevalence in mature animals. The studies produced basic information about the disease. They established some base line point prevalence figures and drew attention to areas worthy of further study, particularly the regular occurrence and re-occurrence of the disease in certain locations, and the predictable age and sex distribution of disease.

Limitations of cross-sectional study design

To understand the way disease behaves in a population requires an understanding of the significance of host factors, e.g. the effects of sex, age and physiological states such as pregnancy, and of modifications induced by external factors such as food availability, weather and social behaviour. To understand the dynamics of the disease in a population, it is necessary to determine the rate at which individuals become infected and for how long they survive. Reliable detailed information such as this may not always be needed if effective control measures are available.

The gradual realisation that the tuberculosis problem was escalating with no real solutions at hand led to the adoption of a structured epidemiological approach to study the problem with an overall aim of improving control methods. A unique five year longitudinal study of the epidemiology of tuberculosis in possums under natural conditions at Castlepoint in the Wairarapa commenced in 1989 (Pfeiffer, 1994).

In that study, the cumulative incidence over 22 months was 11%, a figure which was considered by Pfeiffer to considerably under-estimate the true value. Point prevalences varied from 0.02 to 0.18 and clearly demonstrated the degree of care necessary when dealing with cross-sectional data for point prevalence estimates.

Sexually mature animals were 2.6 times as likely as immatures to show clinical signs of disease but there was no difference between sexes. Pseudovertical transmission was almost certainly involved in several cases involving mothers and their offspring. Necropsy data pointed to excretion of organisms by the respiratory route and via discharging fistulae. Possums which became tuberculous were 1.8 times as likely to disappear (or die) as clinically normal possums. The median time from detection of clinical disease to death was three months for mature animals but the period was much shorter for immatures, which often died within one month, and never survived for longer than 2 months. Tuberculous possums had an overall probability of between 0.32 and 0.69 for surviving for six months and between 0.2 and 0.55 for 12 months after entry into the study.

Four REA patterns of *M. bovis* were reported and they showed marked temporal and spatial clustering within the site and during the period of study. The incidence of clinical disease was associated with adverse weather conditions lagged one month.

Disease transmission

Over the 25 years since the first tuberculous possum was discovered in New Zealand, many people have put forward their personal theories on how the disease is transmitted, and virtually every possible explanation has been put forward at some time. However, until recently, there has not been adequate epidemiological evidence to judge which of the proposed mechanisms were true, and what was the relative importance of various possible pathways of infection. Drawing heavily on the Castlepoint study, Pfeiffer (1994) put forward new tentative explanations of the epidemiology which are summarised here.

Observations from several studies, including Castlepoint, indicated that transmission of infection among possums due to activity and feeding while on pasture was unimportant. Night observations at Castlepoint (Paterson, 1993) showed that possums interacted little while feeding on pasture areas, while studies of the spatial distribution of the disease (Pfeiffer and Morris, 1991) indicated that indirect transmission through contamination of pasture was unlikely. These studies suggested that transmission was linked to the social behaviour patterns of the animals and to sharing of the environment in and around den-sites, where the possums slept during the day.

Aggregation of cases into a cluster was not an unexpected finding for a contagious disease such as tuberculosis, which relies on direct or close indirect contact for transmission. However,

although the highest prevalence cluster at Castlepoint supported a high density of possums, it was not the area of highest population density.

Furthermore, although possums from other areas ranged throughout the study area and shared pasture and feeding areas during their nocturnal activities, they appeared to be at low risk of becoming infected, yet their dens were as close together as in the infected area.

The clustering of cases into small areas over relatively short periods of time indicated that transmission also occurred in small areas of habitat over similar short time periods. Possums living in this study site moved about at night and shared tracks, runs and feeding areas, including pasture, with other possums. There were many opportunities for encounters during these nocturnal activities, but the spatial pattern of occurrence of cases into small clusters did not support suggestions that transmission occurred at pasture or during wide ranging activities.

The best explanation was that transmission among adults occurred principally in association with direct contact between possums in the vicinity of dens, especially during the breeding season(s) when courting, competition among males, and mutual grooming all offered very effective methods for dissemination of infection among possums within a local social group, once infection entered the group.

Indirect transmission may have occurred during sequential use of dens but its relative importance in comparison with direct transmission was unclear. Similarly, sequential territorial marking of the same site and sharing of tracks offered opportunities for indirect transmission.

Den-sharing, outside of the mother-offspring relationship, was recorded at Castlepoint on only two occasions and although it has been recorded at other locations in New Zealand by various observers it apparentlyis not usual. Den-sharing behaviour has often been regarded as an indication of overpopulation, and although it offers a favourable opportunity for transmission to occur, its importance is unlikely to be high overall. At Castlepoint, it seemed unlikely to be important for transmission.

Pfeiffer (1994) also proposed several tentative hypopotheses for future testing, viz.
Maintenance of infection in a local possum population is principally dependent on breeding females and their female progeny (which commonly establish home ranges close to their natal

area). A high proportion of female progeny will be infected and although many will die soon after independence, the survivors will develop clinical disease at variable times in the future, depending on the degree of physiological stress suffered through adverse weather, mating and rearing offspring.

Transmission between adults occurs largely during mating activities and agonistic encounters. New foci of infection arise principally from dispersion of pseudovertically infected immature males and a smaller proportion of infected mature males, depending on time of survival and successful contacts between other possums in the new area.

A SUMMARY OF ECOLOGY OF THE POSSUM IN NEW ZEALAND AND ITS INFLUENCE ON TUBERCULOSIS CONTROL POLICIES

Classification

The system of classification of animals is based on anatomical and physical characteristics. The possum suckles its young and has mammary glands, features which put it into the very large class, *Mammalia*. The possum foetus is born at a relatively very immature stage of development after spending a brief time in the uterus. Immediately after birth, it makes its way to the pouch. This feature of a very short gestation time led early workers to believe that the animal did not develop a placenta. It was placed with all of the other pouched animals, marsupials, in a subclass of animals known as the *Metatheria* [*meta*, (Gr), between; *ther*, (Gr), a wild animal]. There are two other subclasses of mammals, the *Prototheria* [*protos*, (Gr), first] and the *Eutheria* [*eu*, (Gr.suffix), typical animals in a group]. The *Prototheria* have no placenta. There are two Prototherians (or monotremes); the Echidna, or spiny ant-eater, and the Platypus, both of which lay eggs and suckle their young.

The Eutheria have well developed placentae and all of the mammals, other than marsupials and monotremes, are classified into this subclass.

Marsupials are further classified into Sub Orders on the basis of two related characteristics, their diet and their teeth structure. Some marsupials are carnivorous and this feature is reflected in their teeth structure showing adaption for that type of diet. They have a large number of incisor teeth and are accordingly classified as *Polyprodontia*. The herbivorous or vegetarian marsupials, of which the possum is one, have two main incisors and are classified as *Diprodontia*. The sharp incisors have a cutting action while the well developed molars grind the plant material which forms their diet.

The next feature which distinguishes possums from some other Diprodonts relates to its hind foot structure, where the first digit is reduced to a nubbin, and the second and third digits are partly fused together to give two claws on one toe. The fused digit feature was the reason that the possum was originally put into the family *Phalangeridae*. It is at this point that the system of classification exhibits some confusion, because in fact, all of the herbivorous marsupials in

Australasia have a semi-fused second and third digit structure. It is necessary to go back into history to understand the reason for this classification.

Australasia has the richest diversity of marsupial species in the world although there are several other species of marsupials in North and South America. The first marsupial to be described, the American opossum, was recognised in 1500 AD in South America. The American opossum is similar in size to the Australian possum, but has a number of distinctly different features. It was given the name, *Didelphis*, in recognition of what was thought at that time to be two separate uteri, the internal uterus and the external uterus or the pouch. There are several Didelphid species in the Americas. They are similar in size to the possum but are omnivorous, have a potential fecundity of about 12 young per female per year and have a short life span.

The Australian possum was first described by European naturalists in 1620 A D, when Europeans first discovered New Holland or Australia. Unfortunately, the name opossum was also applied to this animal. An end to the ensuing and lasting confusion between the Australian possum and the American opossum was sought in 1765, when the name *Phalanger* was given to the Australian possum, based on its peculiar phalanger or toe structure in the hind feet. The confusion did persist however, with the name opossum being used by some writers for both marsupials.

The final classification is in the specific, or Linnæn name, which in the case of the Australian common brushtail possum, is *Trichosurus vulpecula (Kerr) 1792* This is the particular possum species which was introduced into New Zealand, although in Australian there is a large variety of other possums. The pointed nose and long ears and in particular, the brushy tail, were considered to be similar to those features in the fox. An early name was the vulpine possum, vulpine referring to foxlike. Kerr was the name of the naturalist who first described this particular possum in detail in 1792. Other names which have been used at various times are *Phalangista vulpina*, opossum and common brushtail possum or opossum. Troughton (1954) recorded seven subspecies of *T. vulpecula*, including *T. arnhemensis*, in addition to *T. caninus* and *T. fuliginosus*. *T. fuliginosus* was the name first given to the Tasmanian possums but a more recent classification (Smith, 1984) recognises three species, viz., *T. vulpecula*, (Common Brushtail Possum), *T. caninus*, (Mountain Brushtail Possum) and *T. arnhemensis*, (Northern Brushtail Possum). The irritating confusion between possum and opossum has hopefully ended now, with the term opossum being reserved for the American Didelphis, and possum for the

Australian animal. This convention for usage was recommended at a symposium on marsupials in New Zealand in 1981, and the confusion should now only persist in the historical literature. The term possum should in future be the only one used in relation to the species in New Zealand.

Introduction into New Zealand

The history of the introduction of the possum to New Zealand could well serve as a model which clearly illustrates the dangers of liberating an animal with strong adaptive characteristics into a susceptible environment for reasons of short term financial gains.

The first introduction of possums to New Zealand was probably at Riverton between 1837 and 1840, and this and subsequent introductions from Australia and liberations at new sites of New Zealand bred animals have been documented by Pracy (1974).

Pracy also recorded the gradual development of public dissention about possums and the reaction of governments from the initial perception of a valuable resource to its current status as a serious pest.

The first introductions to New Zealand were Tasmanian blacks which were selected for their highly valued characteristics of long dense dark fur and large body size. Possums are widespread throughout Australia and occupy a larger area than any other marsupial. Later introductions from Australia came from various regions other than Tasmania, a strategy which allowed interbreeding of distinct strains, and it is reasonable to assume, from analogy with other animals, that genetic variability and fitness increased.

More introductions were made in the intervening years but the main introductory phase was between 1890 and 1898. This period also coincided with a major release phase of locally bred animals by the Acclimatization Societies. Interest and action at that time was a response to the high skin prices prevailing at that time. Pracy (1974) refers to a letter sent from T.C. Plante of Melbourne to the Premier of New Zealand in 1891, which quotes prices of up to 8s. 3d. each for Tasmanian skins at an 1890 sale.

The period from 1895 to 1906 saw strong Government support for the introduction of possums. This interest resulted in the release of Tasmanian blacks in the Westland, Buller and Grey

districts, within and near the constituency of the Rt. Hon. R.J. Seddon, who apparently took a personal interest in the establishment of the animal.

The next era up to 1940 saw an increasing demand for grey skins with a parallel drop in the value of darker skins. It was during this period that numerous legal and illegal liberations were made, resulting in the animal becoming much more widespread throughout the country. Early in this period the demand for skins must have been high, because legislation was introduced in 1911 which made it illegal to hunt, trap or destroy possums. This protection was removed by Gazette Notice in the following year, only to be re-instated in particular regions over the next few years.

Early concern

It was during the period of spread in the early part of this century that growing concerns about the damage that possums were causing started to be heard, particularly from settlers and fruitgrowers. The Government response to this mounting controversy was to instigate what would now be termed an environmental impact report. Professor H. B. Kirk was asked to investigate the subject of environmental damage and the advisability of further releases. His conclusion in his report in 1920 was that the return from the sale of skins outweighed economic damage to orchards and gardens and that liberations in most districts would be advantageous. He further recommended a restricted open season, a license fee and a royalty on each skin.

Opposition to the possum on the grounds of damage to native fauna and flora and horticulture continued to gain ground however, with the result, in 1947 of the removal of all controls on the taking of possums and the prohibition of further releases.

The next significant date was 1951 when a bounty system of 2s. 6p. per head cape was introduced as a interim control measure. This was in recognition of the fact that the Department of Internal Affairs did not have adequate methods and sufficient resources at that time to effectively deal with the problem of control of the pest. The Department did however assume the responsibility for control and continued with its own control operations. It is well recognised that the scheme was not successful although it persisted for 10 years to 1961.

A total sum, slightly in excess of £1,000,000 was paid out, much of it for easily accessible possums, including those killed on roads. During the period from 1951 to 1961, 8.2 million

possums were accounted for under the bounty system and a further 4.3 million skins were exported. Despite this activity, possums continued their dispersion into unoccupied habitat in many parts of the country.

Reasons for success

The possum has proved to be a very successful and resilient immigrant in the New Zealand environment and it is a worth-while exercise to examine the reasons for such a dramatic success. Kerle (1984) points out that *T. vulpecula* is the most widespread marsupial in Australia (he includes in this the northern distributed *T. arnhemensis*, which he does not consider a separate species, but *T. vulpecula* alone has an exceptionally wide distribution).

A wide geographic distribution and an ability to colonise diverse habitats ranging from suburbia to a wide range of forest and bush types indicates a high level of adaptability. Early trap mark release studies of the possum in Australia by Dunnet (1956; 1964) and How (1972) provide some of the reasons for this remarkable adaptability. How (1972) made comparisons between *T. vulpecula* and *T. caninus*, the Mountain Brushtail Possum, which is confined to a much more restricted range and may therefore be considered less successful or adaptive than *T. vulpecula*. He concluded that the most important mechanisms for success were high fecundity coupled with early dispersal of the young, facilitating rapid colonisation of new habitats. There are quite a few other species of possums in Australia, but the others all have limited habitats and show no marked propensity to colonise new habitats.

The New Zealand habitat, with its abundance of acceptable and nutritious food sources, allowed full expression of these traits . An added bonus was a lack of competition from other animals and very limited predation. Possums in New Zealand are able to regularly den at ground level, whereas those in Australia are largely forced to den in trees, making den-sites a much more limiting resource. Colonisation in New Zealand may have also been made easier, as suggested by Pracy (1974), through deer browsing at ground level and opening up forests to allow easier movement of possums.

When favourable habitats are separated by unfavourable areas, the ability to disperse to new locations is influenced by the distance between such habitats and the existence of corridors between them, along which dispersing animals may travel. Such corridors are common in the New Zealand countryside. Brockie *et al.* (1987) plotted the frequency of possum road casualties

along a well travelled highway and found that these casualties were most frequent close to waterways vegetated by willows or scrub. This indicated that possums were regularly traversing the highway at those places. The findings also drew attention to the possibility that such corridors might be ideal for the dispersal of possums infected with tuberculosis.

A successful introduced animal such as the possum will normally go through an early proliferative stage during which numbers increase until a peak density above the true carrying capacity is reached. This peak density can be expected to be maintained for a variable period of time before the population inevitably starts to decline to a lower stable level. At this final level, fluctuations in numbers do occur but they are more in the nature of short term variations around an average density, termed the carrying capacity.

Ecologists have yet to conclusively determine what factors limit the numbers of small herbivorous animals such as the possum. Gibb (1981) proposes the hypothesis for small animals in general, that food shortages of varying degrees are of prime importance by directly or indirectly predisposing certain animals in a population to mortality from a variety of causes. Thus food shortages and relative overcrowding may induce social changes in a population which in turn lead to stress-related deaths and poor reproductive performance. While these factors may be significant in the possum, availability of den-sites and adequate protection from adverse climatic influences seem to be at least as important (Green, 1984).

Basic physiology of the possum

An understanding of some basic physiology of the possum is helpful for an appreciation of the way the animal behaves and reacts to its environment. This also includes the way an animal may react to a challenge from infection with tuberculosis, how it then deals with the infection and whether the infection progresses to disease.

The possum has been the subject of extensive study in Australia, which is fortunate, because it allows base line comparisons between the volatile New Zealand population and its relatively stable Australian counterpart. A major review for Australia was compiled by Tyndale-Biscoe (1973).

Tyndale Biscoe notes the variation in body weights of the possum in Australia from 1 kg in the tropical north to 4 kg in Tasmania (characteristic of species which occupy a wide range of temperature zones) and from dark grey coat colour in the tropical north to dark red in the south. In Tasmania dark coat colour occurred more frequently in areas of high rainfall.

Body size and energy loss

Possums have a small body size with a relatively large surface area. As the surface area per unit body weight of an animal increases in line with decreasing body size, the opportunity for energy loss through the skin increases. Energy losses can only be replaced through food supplies and the animal's digestive tract correspondingly is designed to accommodate such losses and make the best use of food resources in its habitat. During periods of favourable weather accompanied by ample supplies of nutritious food, animals have the opportunity to be in a positive energy balance and to lay down fat reserves and increase body weight. Conversely, when a small animal suffers from a decline in food supply, it becomes at risk from energy loss very quickly. Energy loss is a serious event which often leads to death and this has particular relevance to possums in winter when the energy loss may be compounded by inadequate shelter from cold, a reluctance to forage during both short and prolonged periods of inclement weather and a lower nutritive value of available food.

Possums are considered as herbivorous animals although there is evidence that possums prey on and scavenge bird eggs, birds and mammals (Morgan, 1981; Brown *et al.*,1993). Clout (1977), Suckling (pers. comm. in Kerle, 1984) and Cowan and Mooed, (1987) examined possum faeces collected over 4.5 years in the Orongorongo podocarp/broadleaf forest and found that stick insects, cicadas, wetas, beetles and dipteran larvae were eaten regularly, but generally comprised only a small part of the total diet. Possums are close to the lower limit of body size for herbivores, and apart from times when they are feeding on fruits, they require a continuous daily intake of a large volume of food which is metabolised relatively slowly. In this regard they are at a disadvantage compared with small carnivores and insectivores whose digestive systems are designed for rapid conversion of food to energy.

Tyndale Biscoe (1973) records the basic digestive system as comprising a relatively small simple stomach with a pH of 3-4, with a larger caecum with a pH of 8.5-9, apparently acting as a hind-gut fermentative chamber for cellulose digestion. This is a similar system to that found in rabbits. The natural diet of herbivores is high in cellulose which is degraded by the action of

microbes in the gut to short chain volatile fatty acids. This process takes some time and requires a large expansion of the gut to hold the ingesta while the cellulolytic microbes do their work. This expanded gut may be at the start of the digestive system as is the case with the rumen in sheep and cattle, or at the hind part of the gut, as is the case with the possum and the horse. As Hume (1978) points out, the advantage for the fore-gut fermenters is that the microbial protein and B-vitamins manufactured in the fore-gut can be utilised via the small intestine. The disadvantage is that readily digestible starches and sugars are also fermented with some energy loss. In the hind-gut fermenters, these components may be utilised in the small intestine but much of the microbial protein and vitamins may be lost in the faeces. The possum can probably utilise both the small intestine and the hind-gut for digestion and probably varies utilisation depending on the type of food available at any time Fruit would be digested mainly in the fore-gut while leafy vegetation would be processed more slowly in the caecum. Unlike rabbits and hares, possums do not exhibit coprophagy.

Fitzgerald *et al.* (1981) recorded full and empty stomach weights relative to total body weight as 3% and 1.4%, although when feeding on ripe karaka fruit, the full weight may reach 300 gm (personal observation). By comparison, the combined caecum, colon and rectum comprise 3% empty and their contents 6%. Apparent digestibility of cellulose was about 30% and for hemicellulose, 50 to 70%. The ability to deal readily with a range of diets is another indication of an animal which can take advantage of and adapt readily to diverse situations.

Although the possum has this ability, it also very susceptible to energy loss situations. Its dense fur coat is protective and insulating but it is not waterproof. Ward (1977) noted that possums were reluctant to venture out of their dens when it was raining hard or when the forest canopy was dripping heavily. Live trapping studies also show reduced catch success on wet occasions (Bell, 1981; Cowan, 1987). Bell's observations indicated that low winter body weights are probably related not only to a decline in food availability but also to winter adversity. During the Castlepoint study, Pfeiffer (1994) noticed that possums in poor body condition, which were caught nightly for 5 nights during catch release studies, suffered from the combined effects of starvation and exposure and occasionally died.

In fine weather, brushtail possums maintain a body temperature of 36-37^0 C. when ambient temperatures are in the 10^0 C. to 30^0 C. range, and above that temperature they restabilise at 39^0 C. (Tyndale Biscoe, 1973). Cooling is by panting and the arterio-venous loops under the

mucosa of the trachea as described by Tucker (1972) are probably a special adaption for this purpose. Most marsupials have a comparatively low basal metabolism and *T .vulpecula* is no exception, with a value of about 70% of that of eutherian mammals (Tyndale-Biscoe, 1973).

Ecology

The possum is a relatively easy animal to study in the wild state. Its importance as a pest in New Zealand and its asset value in Australia have given it some priority among naturalists as a subject for research. It is easy to study because it is relatively docile, readily trapped and is widespread and accessible to research workers. Despite the attention it has received, there are still gaps in the understanding of its population dynamics, a subject of vital importance to agencies involved in possum control.

Population dynamics

A number of studies have been made of the population dynamics of this animal in Australia and New Zealand. Dunnet (1956; 1964), Tyndale-Biscoe (1955), Crawley (1973), Clout (1977), Bell (1981), Clout and Efford (1984), Brockie *et al.* (1987), Green and Coleman (1986), Jolly (1976), How (1972; 1981), Winter (1976), Triggs (1982), and Pfeiffer (1994) have all reported on this subject.

These studies have used capture/mark/recapture methods wherein the animals are trapped at regular intervals with traps set in fixed positions in a manner intended to cover the whole population in a given area. The animals are permanently identified and regular measurements are made of various parameters to give indices of growth, reproduction and mortality. In addition home range and population density estimates can be made, and the social organisation and aspects of behaviour, including the dispersal phase of young animals, can be studied.

Possum populations have been studied intensively in only a few habitats in New Zealand although numerous observational studies of particular aspects of the species have been made throughout the country. Findings from studies in different habitats and over the four seasons give indications of the effects of environment on those characteristics which are inherent in the population. A number of aspects need to be carefully studied to understand the structure, organisation and dynamics of a population.

To describe the general make-up of a population information is required on its age structure, sex ratios, individual body sizes and population density.

For a description of population dynamics, information is needed on longevity, along with natural and induced mortality, growth rates within the population, reproductive ability which includes the breeding performance of various age groups, and the gains and losses from dispersals.

Additional information comes from measurements of food availability and the effects of seasons and weather.

The description of the population is completed by studies of individual and group social behaviour. Physical factors, such as availability and nature of den-sites and the presence of predators, need to included for study because of their influence on patterns of behaviour.

General population structure

Age structure

A stable population may be described as one in which the number of animals is constant over the long term, with births equalling deaths, gains from dispersals equalling losses from dispersals, and also wherein many individuals are able to live the full term of their natural life span. New populations, which colonise successfully, initially go through a proliferative phase during which numbers increase exponentially. A period of adjustment follows, during which numbers may decrease to a final stable phase. At this stage, any fluctuations might be expected to be minor, leading to the maintenance of a relative state of equilibrium with the environment.

Tyndale-Biscoe (1955) drew attention to the differences in the percentages of immature females between two populations of possums. He noted a higher proportion of immature animals in a population which had been subjected to trapping in the previous two years compared to the proportion in a relatively undisturbed population. It should not be concluded from this finding that a preponderance of young animals indicates a proliferating population, because other factors need to be taken into account before such an inference may be drawn. Brockie *et al.* (1981) examined age data from 14 different populations and concluded that age estimation of possum populations is of little value in deciding whether a population is on the increase or decrease. The authors listed age-specific death rates, the incidence of double breeding and spring births, the

migration or dispersal of certain age classes, overwhelming degradation or improvements in the habitat, control operations, food crop successes or failures and perhaps predation or disease as factors which shape the pattern of age structure. In addition they pointed out sources of potential bias inherent in most sampling methods.

A comprehensive ecological study of a non-tuberculous possum population has been conducted in the Orongorongo valley in New Zealand since 1965 to the present and has provided useful baseline data about certain aspects of the ecology of the animal.

The average annual mortality rate for the Orongorongo population is approximately 15% and the mean life expectancy is about 6 years. The average median winter age of this population between 1970 and 1990 was 4.5 years for females and 3.5 years for males (Efford, 1991). Occasional individual wild possums will live to 12 years of age (Crawley, 1970). Possums can be aged on the basis of the deposition of cementum layers in the molars (Pekelharing, 1970; Clout, 1982).

Sex ratio

When Hope (1972) combined data from a number of studies of possums at 14 different localities in Australia he found that there was an excess of pouch-young males over females at birth (36 males, 16 females). A differential mortality rate caused this difference to disappear by the time the pouch-young were 100 to 150 days old. Hope also noted an even sex ratio in most adult populations, but recorded one sample from South Australia with a highly significant excess of males (202 males, 92 females).

Brockie *et al.* (1981) pooled samples from 9 populations in New Zealand and found a male-dominated ratio in the 0-1 year class with 135 males to 100 females and a similar domination in the two year old class with a ratio of 124 males to 100 females. The ratio was close to parity from 2 years to 9 years, after which there was a swing to a ratio showing a preponderance of females. Brockie *et al.* (1987) analyzed data from a long term study at Bridge Pa in Hawkes Bay and found no differences between numbers of males and females related to location within the study site or to season.

Brockie *et al.*(1981) considered that large variations from parity in the data they used may have arisen from sampling bias because young males may be more easily trapped, poisoned or shot

when they are actively dispersing. Coleman and Green (1984) also drew attention to possible sources of bias when trap-recapture or kill-trap studies are used as a method for estimating sex ratios. They had recorded unbalanced sex ratios in their live trapping study at Mt. Bryan O'Lynn but this same population showed sex parity when they trapped it to extinction.

Several reasons were put forward in explanation. Males were more likely to be caught because mature males moved over larger areas than females and immature males covered large distances in the dispersal phase. It followed that males encountered more traps and depending on the trap layout, may have been over-represented in trapped samples. On the other hand, Coleman and Green (1984) found that females were apparently more prone to retrapping and appeared to be less trap-shy than males.

Clout and Efford (1984) depopulated a 24 ha area by poisoning and monitored the recolonisation. The re-establishment was rapid and after 12 months had reached 50% of the original size. At that point in time, young males were predominant and the male-female ratio was 2 : 1.

Clout and Efford (1984) also examined dispersal in three separate New Zealand environments. Young males dispersed over longer distances than did females and very few males finally settled close to the area where they were reared. On the other hand, young females usually establish in areas close to or overlapping the home range of their mother. This finding of male domination in recolonisation led Green (1984) to comment that recolonisation may have been a factor in some of the populations which were examined by Brockie *et al.*(1981), since some of them had been previously trapped and poisoned.

Green and Coleman (1984) monitored the recolonisation of a population which had been trapped to extinction and confirmed the early colonisation by immature males recognised by Clout and Efford (1984). They found that increases in the first year were entirely due to dispersals. Of the approximate doubling of numbers in the second year, 75% of the increase could be attributed to dispersal and the remaining 25% to local births. At the same time, the low density in the colonising population resulted in better physical condition in the early colonisers leading to better reproductive rates in the new population compared with the pre-kill population.

Body size

Body size shows considerable differences between populations and such variation may be due to genetic and environmental effects. Adult weights range from 1.2-1.9 kg in the north of Australia up to 3.8 kg in Tasmania, and this variation probably represents local adaptation to the effects of environmental temperature. Specific studies to define the separate effects of environment and genetic make-up have not been done. Genetic effects are likely to be of minor importance in New Zealand where the various populations are relatively recent, are derived from diverse introductions, and have been modified by subsequent releases Pracy (1974). Mean body weights vary between locations in New Zealand. Jolly (1976) recorded 3.5 kg for Banks Peninsula, Crawley (1972), 2.6 kg for adult males and 2.33 kg for adult females in the Orongorongo valley, Fraser (1979), 3.58 $\pm$ 0.67 kg for a moderate density area and 3.27 $\pm$ 0.57 kg for a higher density area in the Copland Valley, Clout and Gaze (1984), 3.24 $\pm$ 0.41 for males and 3.03 $\pm$ 0.37 for females in a New Zealand beech (*Nothofagus*) forest at Mt. Misery in the Nelson Lakes National Park. The weights recorded separately by Bell (1981) for the Orongorongo valley were virtually the same as those recorded by Crawley (1972). Cowan (1991) has produced a more extensive list of recorded body weights from the many studies undertaken in New Zealand, although comparisons between populations on that basis alone cannot be easily made from the data..

Within populations, body weights show seasonal variation and a variation in the pattern of the weight changes is evident between the sexes. This has been demonstrated in a number of long term capture release studies. Bamford (1970) devised a method for estimating body weight and condition changes by developing an index of standard weight based on the relationship between body length and weight. He was able to show, for the population he studied, that deviations from this standard weight reflected changes in the proportion of body fat to total body weight. He also demonstrated a relationship between time of the year and fat reserves. A similar correlation between body length, weight and amount of body fat was demonstrated by Fraser (1979). The use of the standard weight index facilitates the study of relationships between body weight and other population parameters such as fecundity, density and mortality.

Bell (1981) analyzed data collected between 1966 and 1977 from a live trapping study of possum populations in the Orongorongo valley and was able to show a strong correlation between changes in female body weight and annual variation in breeding productivity. In those years when mean body weights of females were higher, births tended to be earlier in the season, more

females bred and more of their young survived. Green and Coleman (1984) confirmed this link between better breeding and higher mean weights of females in their recolonisation study at Mt. Bryan O'Lynn in Westland between 1978 and 1981. The first recolonisers of the depopulated area were young dispersing animals. By the time they were 4 years old they were heavier than their counterparts of 1978 and a higher proportion of them had bred. Depopulation resulted in better food supplies for incoming animals, leading to larger body size and higher fecundity. This presents a dilemma for short term and small area possum control operations. The population sink which results from the kill operation can be rapidly recolonised by a combination of immigration and more successful breeding among immigrants and the residual population.

Population density

Population density is recorded as the number of animals per hectare and is most accurately derived from intensive trapping or poisoning studies. In those areas where there are well established populations, the quality and abundance of food together with the availability of den-sites are probably the most important factors which regulate density. Density may be further modified by natural influences such as floods, droughts and intervention by man in poisoning or trapping operations.

Batcheler and Cowan (1988) listed density statistics for various habitats in New Zealand. Broadleaf podocarp forests varied from 7 to 10.7 possums per hectare, beech forest was low at 0.5 per hectare, pine forests 1 to 3 per hectare and farmland 0.5 to 1.2. However, within those habitats there was considerable variation. In the Westland broadleaf podocarp forest studied by Coleman *et al.*(1980) an overall density of 10.7 was recorded but they were able to further stratify this figure by taking altitude and forest-pasture association into account. They found highest density (25.4 possums per ha) at the forest margins and the lowest (1.9 per ha) at the 800 to 900 metre altitude zone.

Similarly, while Brockie (1991) recorded an overall density of 1 possum per hectare on farmland in Hawkes Bay, he found densities of 8.5 per hectare in a scrub-covered swamp and 16.6 in willows bordering a farm drain within that farmland.

The first estimate of 46 million for the total New Zealand population in 1946 was made by Pracy (1981) who conceded that it was only an educated guesstimate. Brockie (1986, cited by Batcheler and Cowan, 1988) at a later date calculated a total of 63 million and Batcheler and

Cowan (1988) put their estimate at 68.8 million. Their calculations were based on density estimates for various habitats and the estimates of the area of those habitats within New Zealand. While the method of calculation of these total populations has certain limitations, it leaves no doubt that the possum is very common and widespread, and is one of the most abundant mammals in New Zealand.

Four nation-wide surveys of possum populations were carried out between 1948 and 1980, (Pracy, 1980). Currently, there are only a few remote areas in New Zealand which have not been invaded by possums. Some areas, such as the southern half of the North Island, have been colonised for a relatively long time, while in large areas of the northern part of that island, the main colonisation has been within the last 30 years. The stage of development of a population is difficult to assess, but Pracy (1980) made some mainly subjective comparisons of populations throughout New Zealand between 1950 and 1980. He concluded that there had been a steady increase in the number of areas showing declines from peak population densities over this 30 year period.

Reproduction

Adult female possums come into oestrus in the autumn and if not mated they will continue to cycle until late spring. During summer they either do not cycle or cycle irregularly. The length of the cycle varies around a mean of 25 days (Tyndale-Biscoe, 1973) with oestrus lasting up to 24 hours. It is only during oestrus that females will accept a male.

Tyndale-Biscoe (1973) described the development of possums from birth to independence. A pregnancy of approximately 18 days follows a successful conception. At birth, the newborn weighs only 200 mg, yet it makes its way, unaided by the mother, to the pouch by climbing with its forelegs up through the fur. This remarkable passage only takes 3 to 5 minutes and once in the pouch the newborn quickly attaches itself to the teat. The teat swells within the mouth with the result that the young can only be removed with difficulty. At the back of the mouth the epiglottis is large and extends through the soft palate so that the glottis opens into the nasopharynx. The buccal cavity extends around either side and communicates with the oesophagus. By this means the young animal is able to breathe and suck at the same time. The young possum or joey remains attached to the teat for approximately 70 days during which time the young grows relatively slowly and the sucked mammary gland remains relatively small.

The mammary gland then shows rapid development in size until approximately 150 days and during this period there is also rapid growth of the young. The gland gradually reduces as the young becomes independent. The young leaves the pouch permanently from about 170 days and is usually completely weaned at some time from about 190 days on. The mother may breed again about this time but the weanling often remains in close association with its mother and within its mother's home range for several more months. Bell (1981) found a positive correlation between the weights of the immatures at 170 days, which varied from 240 gm to 440 gm, and the mid-winter maternal body weight. Those females which had low mid-winter body weights reared offspring with low 170 day body weights.

Bell also found that adult females which had relatively high summer weights and continued to increase in weight during the autumn were the most successful breeders. Conversely, those females which had no young generally had relatively low body weights in the summer and showed a decline in weight into the autumn. Females which had offspring, but were unsuccessful in rearing them, tended to fall between these two groups.

Bell made his observations in the Orongorongo valley population in which breeding principally occurs in the autumn. Kerle (1984) listed the monthly distributions of births for the known studies up to that time. In New Zealand most of the births are during the April to May period, with a lesser birth pulse in October to November. The heavier females are the earliest breeders and in those years when average female body weight is high in the autumn, the breeding season is early.

The ability to breed again in the spring is almost certainly related to the seasonal availability of highly nutritious food for the lactating female and the newly emerging young. This is the most likely reason for the observed regional differences and differences between years that have been recorded in New Zealand for this secondary breeding pulse. Spring births are almost absent in established populations in indigenous forests, such as the Orongorongo. On the other hand, they do occur in situations where food is plentiful and the population is colonising, and also in the bush-pasture habitats of established populations, such as at Castlepoint (Pfeiffer, 1994) and Bridge Pa, (Brockie *et al.*, 1989).

Green (1984) compared breeding rate by age class in two locations in New Zealand and one in Australia. Females at the end of their breeding season as three year olds in Australia have weaned on average 2.5 young for their lifetime to date. In New Zealand by the same age they

had weaned two young if they were in a privileged colonising population or occupied a bush-pasture habitat. Those females in established populations in a forest will have only weaned 1.5 young. These calculations assumed an 85% survival of pouch-young to weaning.

The annual increase in the size of a stable population is balanced by losses from deaths and migration. The undisturbed Orongorongo Valley population has been studied intensively for 24 years and fluctuates annually about a mean population density of 8.2 possums per hectare. Within that time it has shown extremes of 5 and 13 per hectare. Ecologists consider this possum population to be an example of an exceptionally stable wild animal population. Efford (1991a) examined the reasons for the annual fluctuations and points to the highly variable number of one-year-olds in the winter population as being one important cause. The other factors, autumn female body weights and the production of fruit by hinau trees (*Elaeocarpus dentatus*) are correlated with the proportion of the females breeding and the survival of their young.

The Orongorongo population regulates itself about an equilibrium and Efford (1991a) recently produced evidence that suggests that the important factors controlling this balance are winter mortalities and immigration of possums over one year old. Efford also drew attention to the low body weights of yearlings in this population at 58% of adult weight compared with 80% in the possums on Hawkes Bay farmlands reported by Brockie *et al.* (1989). Low autumn body weights of the Orongorongo juveniles probably predispose them to death during the winter.

Home range, dispersal, emigration and immigration

Migration of young animals away from their parents' territories is a common inbuilt characteristic in many wild mammalian species. This natural tendency is an important mechanism for the outward spread of possums into new or depleted areas and also has strong implications for the spread of tuberculosis if the migrating or dispersing possum is carrying disease.

The home range for a possum is defined as that area to which it confines most of its activity and wherein it spends most of its life. The extent of home ranges of individual possums can be calculated from trap release and radio telemetry studies. Home ranges estimated by trapping are smaller than those estimated by radio-tracking. Forest dwelling possums have home range sizes usually from 1 to 3 hectares but Brockie (1991) recorded much larger values, from 2 to 105 hectares, (mean = 30 ha), for possums in open farmlands in the Hawkes Bay.

In radio telemetry studies, a small radio transmitter attached to the possum allows observers to follow the movements of the animal by monitoring the location of the radio signal. Home ranges overlap and are shared, but the limited size of these home ranges means that the adult population is relatively stable as far as movement is concerned. Adult males tend to wander further than do females and accordingly have larger home ranges (Green, 1984; Brockie, 1991; Paterson, 1993). Both sexes may occasionally move outside their main area of activity to feed on highly palatable foods or special trees when fruits are ripe.

The dispersal phenomonem is first seen in a proportion of young possums when they are about ten months of age. They have become independent from their mothers at about eight months of age, usually about December. At about 10 months, around February, some will disperse, although many will remain close to their natal area. The physiological basis for this behaviour is unknown, but they will travel considerable distances in a short time, such as 5 km in two nights (Ward 1985). Brockie (1991) records one animal travelling 10 km in two weeks and documented one female which travelled a total of 25 km. Although more males disperse, female dispersers tend to disperse over greater distances than males and will often make several moves, presumably in their search for a suitable habitat for permanent settlement. Studies in New Zealand have generated data on 69 possums which are known to have dispersed at least 2 km, (Efford, 1991b). The median long-distance dispersal distance was 3.7 km for males, (up to 11.6 km), and 5.5 km, (up to 41 km), for females. Most dispersers are males, outnumbering females by 4.3 to 1. A second phase of dispersal is seen in spring and summer when the animals, at about 18 months of age are starting to show signs of sexual maturity. This phase has generally received little attention but Efford (1991b) considered this phase to represent the largest number of dispersals in the Orongorongo population. Within this particular population, four of five breeding males and one of three breeding females were immigrants.

If the population is stable, emigration is matched by immigration in this dispersal phase. In the natural state, without intervention from predators or man, the factors that come into play to regulate the population size and stabilise it are the natural mortalities imposed by the stresses of the breeding season and winter lactation, food shortages and cold during the winter, and the stresses arising from uneven competition between young animals seeking permanent home ranges and successful settled adults. When tuberculosis is established in a population, it also contributes significantly to deaths.

Juvenile mortalities

Few of the possums which are born survive to 2 years of age. Some interesting statistics on the fate of 116 pouch-young born in 1979 and 1980 have come from the Orongorongo study.

In this location, young are born almost exclusively in the autumn. Ward (1985) found that of these 116 pouch-young, 39% died in the pouch and 38% disappeared, presumed dead, between leaving the pouch and 9 months of age.

Of the 27 left, 10 died in the study area and 6 dispersed, leaving only 11 of the original 116 animals alive and living in the area where they were born by 2 years of age.

It should be appreciated that the Orongorongo population is a particular case which has been studied intensively for a longer period than any other population in New Zealand or Australia. As such, it has been valuable in providing a great deal of basic information about factors which may influence possum population dynamics. However, the Orongorongo forest habitat has its own characteristics, many of which differ substantially from other habitat types found far more commonly in farming areas. For this reason, the data from this site (or any single site) should not be regarded as absolute across all populations but interpreted in a cautious manner when making predictions about other populations. Only one of 1470 possums tagged and 374 necropsied in the Orongorongo research area was diagnosed as tuberculous, despite the existence of known tuberculous animals in a valley 1.3 to 4 km distant (Brockie *et al.*, 1987).

Adult mortalities

Adult possums in the wild do not have a long life expectancy. Studies have indicated that annual mortalities in adult possums are of the order of 15 to 30% (Spurr, 1981; Clout and Barlow, 1982).

There is a well defined seasonal pattern to these deaths. Most of them occur in the winter when animals are exposed to cold wet weather and deprivation of quality food supplies. Possums show a reluctance to leave their dens to forage when it is raining (Ward, 1978) and the resulting drop in food intake at a time when cold weather is imposing extra demands for energy can result in death. Immature possums, in particular, have the added burdens of still growing and competing with established older residents for home range, food and shelter.

Moribund non-tuberculous possums are occasionally found wandering aimlessly during the day time in the winter. At necropsy, such possums commonly show emaciation, loss of fat reserves and adrenal exhaustion, indicating severe stress and starvation (personal observation). Some animals die from prolonged environmental and feed stress and show evidence of chronic abnormalities, while other possums die more acutely of exposure, exacerbated by short-term energy depletion.

Data from B.M. Fitzgerald, (cited by Efford, 1991), showed that the proportion of cat scats containing possum remains increased markedly during the winter. Feral cats feed on possum carcasses and this finding gives some additional support to the seasonal nature of possum deaths.

Tuberculosis can be a significant cause of death in populations which harbour this disease, and its incidence and mortalities exhibit a definite seasonal pattern of occurrence (Pfeiffer, 1994).

A number of diseases have been reported in possums, but of those, only tuberculosis has been thoroughly studied in a wild population over time. Consequently, the importance of such diseases and whether they cause significant losses is not known. The subject of parasites and disease of possums has been reviewed by Presidente (1984) and more recently by Wilks (1990). Apart from tuberculosis, the only other serious disease which commonly leads to death is liver fluke infestation. Possums are susceptible to infection with liver fluke (*Fasciola hepatica*), a common parasite of sheep and cattle in some regions of New Zealand. Relatively few infective metacercariae, (the infective stage on the pasture), are required to cause severe illness and death.

Winter starvation is in itself a significant cause of death, while debilitation from reduced food supplies probably predisposes to other disease complexes, including tuberculosis.

Territorial defence, spacing, and social behaviour

Observations have confirmed a system of dominance within groups of possums and it is reasonable to believe that dominant males actively compete with and discourage other males during the breeding season. Australian studies suggest that successful adult possums actively defend their key resources, viz. food trees and dens, and that this system of defence acts as a population density regulator (Dunnet, 1964; Winter, 1976).

Green (1984) contrasted the relatively low population densities of Australia with the much higher densities of New Zealand and drew attention to the considerable differences between the ecosystems of the two countries. In Australia, the possum lives most commonly in open Eucalypt forests at relatively low densities, of the order of approximately 0.5 per hectare. Safe den-sites in this habitat need to be high above ground to make them inaccessible to predators and are generally confined to hollow branches and hollows within trees. Such den-sites are scarce in Australian forests.

Mackowski (1984) described the process by which hollows form in Blackbutt eucalypts and drew attention to the considerable age of approximately 200 years that these trees have to attain before hollows will form.

The availability of suitable den-sites is much higher in New Zealand than in Australia because a virtual absence of predators in New Zealand allows possums to den with impunity at ground level as well as in trees. Green (1984) argued that in forest habitats in Australia, where den-sites are scarce, population density is principally regulated by the availability of that key resource. Density regulation based on availability of a key resource such as den-sites, as in the Australian situation, is ineffective in situations where there is a surplus of that resource, as in the New Zealand situation. Where population density is regulated, as in Australia, by the availability of den-sites, abundance of food may be a secondary regulatory mechanism. It follows then, that in such habitats, severe or prolonged depletion of food sources may be needed to influence population density.

Green (1984) pointed out that on the other hand, if density is not controlled by den-site availability, as in most new Zealand situations, food resources become more important. Short term depletions of food resources are more likely to affect reproductive performance or even mortality in such circumstances and act as important regulators of population density. If the shortages occur during cold or wet periods, then the outcome for disadvantaged members of the community, such as juvenile animals, may be serious.

Den-site usage varies between the two countries. Winter (1976) found that possums in his study area on the outskirts of Brisbane used 2 to 5 dens per year per animal. Den sharing occurred between males and females during the consort period leading up to and during mating and also

by mothers and their offspring. Specific dens were not used by different mature animals of the same sex. How (1981) also found that specific dens were generally used by one possum.

In New Zealand, Ward (1978) studied possums in the Orongorongo study area and found that most of the den-sites were above ground in large trees. Each animal used 6 to 13 dens per year and there did not appear to be competition for dens.

A more extensive study was later carried out by Cowan (1989) who observed 55 possums in the same Orongorongo study area. He recorded a usage of 11 to 15 den trees per possum per year but found 60 to 75% of use was confined to 3 dens. He also re-examined Ward's data and therein found a similar preferential use of several dens per possum. He noted some den sharing in the breeding season and sequential use of dens by different possums. The sequential use usually was restricted to several animals but some dens were used sequentially by up to 9 different possums. He calculated that there was a 50% chance that a den would be used by a different possum within 10 days.

Green and Coleman (1987) found a similar usage of dens of 10 to 15 per possum per year but in their Westland study area, most of the dens were on the ground. In New Zealand, males and females apparently were not inhibited from using the same den sequentially.

The internal dimensions of most dens are small and the interior is usually well protected from the elements. These factors, plus sharing and sequential use, have led to speculation that such sites may be ideal for the survival of *M. bovis,* (Coleman, 1988) and may therefore be an important factor in the transmission of tuberculosis between possums.

Fairweather *et al.*(1987) regularly examined 4 hollow-tree dens and recorded sequential use by a number of possums and occasional simultaneous sharing by several animals, but their observations were made in a very dense possum population of approximately 25 possums per hectare.

Possums at Castlepoint usually den on the ground, most commonly in or under flax bushes, but also in root rakings and under gorse bushes (Pfeiffer, 1994). Den sharing, outside of the mother offspring relationship, appeared to be a rare event. Pfeiffer and Morris (1991) recorded a possum denning on top of the carcasses of dead possums and this is in line with occasional

anecdotal reports of similar behaviour. During three years of that study, there was only one recorded occasion when two live and presumably unrelated possums (both males) were observed together in close contact with one another in the same den (Paterson, 1993).

Behaviour and territorial defence

Possums are generally solitary animals, but many questions remain unanswered about their social grouping and behaviour towards one another. The most complete observational study was carried out by Winter (1976) but his observations were made in a low density population in Queensland and may not apply for New Zealand habitats where densities are generally higher and key resources such as den-sites are arranged differently.

In New Zealand, possums generally do not have exclusive territories but share their home ranges with others (Crawley, 1973; Green, 1984; Paterson, 1993). Males usually have a larger home range than females (Green, 1984; Paterson, 1993). It is reasonable to assume that possums have a system of territorial defence, although territories are certainly not exclusive ones and there must be some subtle definition of territoriality which forms the basis of social relationships. Their pattern of selective den usage and the difficulties that young possums experience after independence support defence mechanisms by older dominant animals. How vigorously they defend territory is a matter of conjecture, although Cowan (1991) reports that occupied dens are defended. Bite or claw wounds have the potential to be a route of infection for tuberculosis but the pattern of occurrence of such injuries has not been studied. Wounds are occasionally noted in possums and agonistic encounters certainly occur during mating, when up to six males may cluster about an oestrous female (Ward, pers. comm.; Sauter, pers. comm.). The significance of such events in relation to transmission and occurrence of tuberculosis remains unknown, but close agonistic encounters provide potential opportunities for aerogenous transmission of respiratory infections.

Territorial marking may have relevance for indirect transmission of tuberculosis. Possums commonly mark objects with their chin and sternum (Biggins, 1984) and presumably leave information to other possums via the secretory glands in this region. They also mark trees extensively by bark biting. Both of these methods have a potential to act as mechanisms of transfer of infection. Tuberculous possums commonly have discharging lesions in the axillary region and under the chin. Sequential rubbing and marking behaviour by different possums at the same location provides opportunities for transfer of *M. bovis*. Similarly infected saliva might

be deposited on trees during bark biting episodes. The significance of these marking behaviours is presumably for individual recognition and territorial boundary marking purposes.

Possums usually present a well groomed appearance and spend about 11% of their time outside of the den in self grooming (MacLennan, 1984). Biggins (1984) describes the methods of face washing and grooming used by brushtail possums and draws attention to the extensive use of saliva in both of these activities. He considers that saliva performs a secondary function of self anointing the animals with its own salivary odours, which then conveys information to others about identity and perhaps physiological state. Whether mutual grooming is practised outside of the mother-offspring relationship is not known.

Possums spend approximately 16 hours of each day within a den-site. During their nocturnal activities they spend up to 46% of their time immobile (Ward, 1978; Winter, 1976; MacLennan, 1984), 16% of their time feeding and 30% travelling (MacLennan, 1984). Most of these observations were done in Australia and their relevance to patterns of behaviour in New Zealand habitats is not known.

The destructive nature of the animals on the environment has focused attention on the diet of possums and this has led to extensive study of this subject. In an early report on diet, Pracy and Kean (1949) listed over 70 species of indigenous trees, 20 ferns and some vines and epiphytes as well as grasses, forbs and sedges which possums utilise for food. Since that time, further additions have been made to this list (Green, 1984). The effects of possums on indigenous vegetation, crops and pastures, catchment protection, and exotic forests have been extensively reviewed by Batcheler and Cowan (1988).

Possums graze and browse selectively and show marked preferences for some species to the extent that overgrazing of such species has caused them to become very rare in some habitats. The loss of preferred species has not yet seemed to impose any restraints on the ability of the possum population to continue to thrive. Other species take the place of lost previously preferred species. Fitzgerald (1976) reviewed the dietary changes which had taken place in the Orongorongo population over the period from 1946 and 1971. The five main species which had made up 46% of the diet in 1946 only made up 5% of the diet in 1971. Selective browsing by possums was responsible for changing the abundance of those species over the 25 year period.

Despite this loss of preferred species, the population density and production parameters remained constant within normal between year variation.

Possums are commonly observed feeding on pasture around the bush-pasture margins and Gilmore (1967) and Harvie (1973) have estimated that pasture species may comprise 20 to 30% of the possum's diet. The animals obviously divide their time between feeding within the bush or forest, and on pasture in those habitats where it is available. The frequency of their visits to pasture probably varies from time to time depending on the availability of preferred species and total food available.

TUBERCULOSIS IN OTHER ANIMALS AND THEIR IMPORTANCE AS SOURCES OF INFECTION

Introduction

In New Zealand, tuberculosis is occasionally encountered in animals other than cattle, deer and possums (Allen, 1991) and when such a finding is made, speculation often follows about the possible role for these other animals in the maintenance and spread of the disease.

The species of animals in which tuberculosis has been found in New Zealand are conveniently considered under two main groups on the basis of their feeding behaviour, viz. herbivores and flesh eaters.

Herbivores

Deer

Introduction

It is known that some free living deer in the wild are infected with tuberculosis. This information comes principally from examination of carcasses at processing plants. It is supplemented by experience with testing of newly captured deer and the occurrence of tuberculosis in farmed deer following the introduction of wild animals. Tuberculosis in New Zealand red deer caused by *M. bovis* was first recognised in a free living wild deer in 1970 at Mokihinui in the West Coast region of the South Island. In the North Island, it was first recognised in 1976 in a wild animal from the Eketahuna County, while the first occurrence in farmed deer was reported in 1978 in the South Island (Hellstrom, 1979; Beatson and Hutton, 1981).

The predominant and most widespread species of deer in New Zealand is red deer, *Cervus elaphus scotius,* which was first introduced into New Zealand in 1851. By 1923, about 1000 animals had been introduced, some directly from Britain, but about two thirds of the imports came from Victoria, Australia. Some of the Australian and New Zealand imports came from wild deer in the Scottish Highlands but most were from established deer parks in Britain. These

deer parks had mainly been established from local deer but also sourced breeding animals from Germany and Denmark. Whether tuberculosis was introduced with the original imports into New Zealand is not known.

Mycobacterium bovis infection has been recognised in several species of wild deer in other countries, (Clifton-Hadley and Wilesmith, 1991). Countries of particular interest to New Zealand include Great Britain, (Gunning, 1985; MAFF Reports, 1985; 1986) Ireland, (Dodd, 1984), Hungary, (Stuart *et al.*, 1988) and Germany and Sweden (Francis, 1958). Tuberculosis caused by *M. bovis* has recently been recognised in imported farmed deer in Denmark and the United Kingdom (Clifton-Hadley and Wilesmith, 1991) and in Canada and the United States.

Of 161 isolations reported from wild deer by de Lisle and Havill (1985), two were from sika deer (*Cervus nippon*) in the North Island, two were from fallow deer (*Dama dama*) in the South Island and the remainder were from red deer. In the North Island the principal areas containing infected wild animals are the Wairarapa and the area about Lake Taupo.

Little is known about the epidemiology of the disease in deer in the wild because of the occasional nature of investigations, but based on the low prevalence of disease found at processing plants, is would appear that the disease is rare in free living wild deer. On the other hand, high prevalences up to 0.39 have been found in endemic areas about Lake Taupo (Nugent, unpublished).

Farming of deer under intensive conditions started about 1970 with herds being established from captured wild deer. Some of the captured animals were sourced from areas which are known to contain tuberculous deer and these animals were probably responsible for the introduction of the disease into the farmed herds. Although helicopter operators preferred to capture animals from areas with no history of tuberculosis, e.g. South Westland and Fiordland, the high level of demand and high prices prevailing during the formative years influenced some operators to capture in known endemic areas.

Some importations have been made of red deer from Europe and wapiti, (*Cervus elaphus nelsoni*), from North America during the last 10 years.

The national farmed deer herd in New Zealand now exceeds 1,500,000 animals.

Pathology

Post-mortem examination of tuberculous animals indicates that the route of infection in wild deer is mainly respiratory, (Livingstone, 1980 cited by Beatson 1985) but the source of infection is uncertain.

Brooks (1984) autopsied 15 naturally infected animals in contact with 36 experimentally infected deer and found most lesions in the lungs and bronchial lymph nodes. Livingstone (1980) cited by Beatson (1985) found the medial retropharyngeal the most frequently affected node, (44.5%) followed by the thoracic sites, (lung 10%, mediastinal 4%, bronchial 6% and mesenteric 14%) in 119 tuberculous deer from 4 properties. Similar frequencies were also reported by Wilcockson (1986) in 76 cases reported at Ruakura.

In experimentally infected red deer, Brooks (1984) found that lesions were granulomatous with a variable margin of large macrophages and giant cells surrounding a prominent central zone of caseous necrotic cellular debris. Numbers of bacilli varied from few to very numerous. Neutrophils were usually present and were considered to be responsible for liquefaction in some lesions. Neutrophils in large numbers were not a common feature.

Abscess-like lesions which discharge to the exterior have been reported occasionally in deer. Bertram (1986) recorded mesenteric lymph nodes draining into the lumen of the intestine. Robinson *et al.*, (1989), reported lesions discharging to the exterior. Exudate from such lesions contains large numbers of infective bacilli and presents a hazard to other deer via environmental contamination (Beatson, 1985).

Epidemiology

The distributions of lesions indicate that deer can become infected by the pulmonary or the intestinal route. However it would be unwise to speculate on the relative importance of either route in deer at this stage of understanding of the epidemiology of the disease in deer. Personal observations from general veterinary practice about circumstances which may have influenced the epidemiology of the disease in farmed deer are presented here:

The national prevalence of tuberculosis in farmed deer has always been low, but some farms have experienced very high prevalences which have occasionally necessitated complete destocking.

The main factor influencing spread of disease between herds has probably been introduction of diseased animals. High demand and high prices for capital stock often meant that disease control was given only minor consideration during the initial stocking phase and later when buying breeding sires. The development of deer farming was strongly influenced by taxation benefits for investors during the developing stages of the industry and this factor contributed to maintenance of high individual animal values.

Until recently, tuberculin testing of deer herds was voluntary. Although a test and slaughter strategy had steady gradual acceptance, the rejection of testing by some farmers together with some poor quality test application and interpretation hampered control in some areas.

The incidence of tuberculosis in deer is declining steadily (Carter, 1992) and the current situation gives some cause for optimism. The efficacy of tuberculin tests has been evaluated and veterinarians have adopted a uniform approach to dealing with reactors. A decreased demand for capital stock has consequently reduced the need for movement of animals between herds.

Many deer farms are small and well contained without permanent possum habitat. The high capital cost of land, fencing and buildings has contributed to this situation. It is difficult to conceive that such farms are at risk from tuberculous possums. Farms which are large and contain possum habitat or are located on bush boundaries are probably the ones most at risk.

A reduction in natural immunity from stress may have increased the susceptibility to infection in farmed deer during the early establishment days. Newly captured deer did not tame easily, facilities were poor and methods of handling were often rough and poorly understood. Many animals sustained serious physical injuries during capture, losses from disease were high and animals commonly showed signs of poor adaption to confinement. Then as now, deer were often housed and managed in buildings with poor ventilation and restricted air spaces, conditions that are ideal for transmission of respiratory infections such as tuberculosis.

A combination of present improved standards of husbandry and regular testing, now that all herds are under test, can be expected to reduce prevalence and incidence. The high herd prevalences of the past should not recur. However, the relative importance of the various causative factors for infection may be expected to change with changed farming practice and lower prevalence.

Overall, the approach to tuberculosis in deer has been pre-occupied with testing methods, with the result that basic pathology, pathogenesis and epidemiology have not been researched adequately.

Because of the rarity of the disease in deer in the wild and relatively few opportunities for contact between cattle and deer, it is unlikely that tuberculous deer pose any significant threat as a source of infection to free-ranging domestic animals.

The role which tuberculous deer may play in infecting possums in the wild is not known. It is difficult to conceive of a scenario in the wild which would allow adequate effective contact, sufficient for disease transmission to occur between the two species.

Scenarios which allow infection from tuberculous deer to possums to occur under farmed conditions can be postulated. Possums are attracted to den in farm sheds and many deer houses offer a combination of shelter and food. If possums and tuberculous deer share the same air space, there will be opportunity for transmission.

It is well accepted for human tuberculosis that transmission is enhanced under conditions of restricted and shared air space and that tuberculosis in humans is primarily a respiratory disease (Langmuir, 1961; Houk *et al.*, 1968; Nolan *et al.*, 1991). The importance of housing as a risk factor for cattle was reviewed by Francis (1958) and he also cites numerous examples in other animals in relation to housing in zoos, in laboratories and in farming situations where animals are housed.

Ecological factors

Wild populations have experienced major alterations in the past 20 years since commercial hunting commenced in 1958-59. The high density populations, particularly in the South Island, were initially reduced by hunters operating in areas which were accessible by vehicles, jet boats and fixed wing aircraft. About 1965, helicopters started to be used for hunting, opening up hitherto inaccessible areas in the Southern Alpine regions. Challies (1990) records that between 1970 and 1980, deer pellet counts declined by 75% in the Arawata Valley, South Westland, the equivalent of approximately 90% decrease since 1965, when aerial hunting commenced. The residual population was reduced to a low level, but was younger and physically fitter with higher fecundity and low natural mortality. Challies estimates that this particular population had a

harvestable increment equivalent to about 35% of the numbers existing before the new season's calves were added. Increased fitness, in conjunction with lower population densities and smaller group size, may be expected to alter the dynamics of tuberculosis within those populations. Not all deer populations have been similarly affected. Most deer in these alpine regions are shot or captured above the bush line but the vast areas of dense bush without open alpine regions in the North Island present more difficulties for control. Continued hunting pressure is dependent on ruling prices of venison as game, the cost of helicopter operation and the rate of harvest.

Deer are opportunistic feeders and shift their home ranges in relation to season and food availability. Stags tend to keep apart from hinds except in the breeding season and occupy larger home ranges. Both sexes of red deer form single sex groups during most of the year. Prior to the intense hunting pressure of the past 20 years, group size could be up to 150 hinds, although groups of 3 to 12 were more common. Stag group sizes were smaller. A result of successful hunting pressure has been a reduction in group sizes.

Yearlings are usually rejected prior to the birth of the new season's calf. Male yearlings usually disperse to new areas but females tend to return and remain in their family group. Young stags have been recorded as moving up to 32 km from their natal area (Gibb and Flux, 1973).

During the establishment phase, stags colonised new areas first, preceding hinds by 5 to 10 years and sometimes up to 20 years. Caughley (1963) estimated the average long term dispersal rate of local breeding populations to be about 1.6 km per year, depending on terrain, vegetation and the expansion phase of the herd. Clarke (1971) estimated that well established herds in favourable habitats have achieved rates up to 11 km per year.

There has been some speculation that deer may transfer disease over large distances but there is no direct evidence to support this suggestion, although dispersal and expansion ability of the above order provides excellent opportunity to carry disease with the migration.

Whether the disease can sustain within wild deer populations in New Zealand in the absence of vectors is not known.

Sheep

Tuberculosis caused by *M. bovis* is considered to be a rare disease in sheep at pasture, with only occasional cases of the disease having been recorded throughout the world. The disease has only been recorded as a flock problem in New Zealand on two occasions when the prevalence became high enough to warrant some intensive investigation. Both of these cases were in areas where there were also tuberculous possums. One farm was in the Buller region (Davidson *et al.*, 1981) and the other was in the central North Island (Cordes *et al.*, 1981). On the latter farm, 281 sheep out of 15,000 were tested using an intra-dermal test. Of 31 sheep which were positive, 26 had visible gross lesions of tuberculosis. On the Buller farm, 108 of 596 sheep reacted positive to the test. Of these, 79 (73%) had gross lesions.

It has been established that sheep are susceptible to infection with *M. bovis* in situations where conditions are favourable for spread of the infection, as in housed animals, (Francis, 1958) or where the challenge is very high, as in the two cases referred to previously. Lung lesions predominate in affected sheep, indicating transmission via the respiratory route.

It is generally considered that tuberculosis in sheep is a rare disease in New Zealand but there appear to have been few investigations to determine the true prevalence. The true prevalence may be clouded by an inability to distinguish gross lesions at autopsy or meat inspection from caseous lymphadenitis, which is a relatively common disease of sheep.

Goats

There are many similarities between tuberculosis in goats and the disease in sheep. In New Zealand, limited investigations of the disease status of goats were made in the mid 1980s when commercial interest in goats was high and there were extensive movements of goats, often from endemic areas, throughout the country. Feral goats were being used extensively at this time as recipients for embryo transfers and in breeding up programmes. Allen (1987) recorded 35 reactors out of 512 tests, with gross lesions in 13 of 28 reactors at autopsy. Sanson (1988) summarised testing data from throughout New Zealand and found a low prevalence of tuberculosis in goats sourced from endemic areas but also found indications that the disease had been spread further into goat herds throughout the country.

The pathogenesis of the disease is similar to that in sheep and cattle with a predominance of lesions in the chest cavity indicating airborne infection via the respiratory route.

Rabbits and hares

Rabbits are highly susceptible to infection with *M. bovis* by inhalation and inoculation under experimental conditions (Francis, 1958). These features led to extensive use of rabbits in tuberculosis research up to the 1960s. On the other hand, although wild rabbits have shared habitats with tuberculous cattle in many countries, there has been only one recorded case of *M. bovis* infection in a free living wild rabbit. This was in a rabbit in Central Otago (Anon, 1980; Gill and Jackson, 1993). Tuberculosis caused by *M. bovis* was recorded in a free-living hare in New Zealand (Cooke *et al.*, 1993). Another tuberculous hare was trapped in the following year about 200 metres from where the first case was trapped (Jackson, unpublished). The second animal was a young heavily pregnant female in good condition. It is reasonable to conclude that neither rabbits nor hares represent any significant risk for maintenance of the disease, although there was some evidence for limited spread in the case of the hares.

Horses

Tuberculosis is probably a rare disease in horses in New Zealand although several cases have been diagnosed at Massey University (Alley, pers. comm.) and lesions found in a horse at slaughter (Julian and Marshall, 1991). The disease in this species has negligible significance as a source of infection for other animals.

Flesh eating animals

Pigs

Feral pigs (*Sus scrofa*) are widely distributed throughout new Zealand. Although classified as a pest, they do have some informal protected status on many properties because of their recreational hunting value. Tuberculosis in feral pigs has been recognised in many endemic areas but the disease has received limited attention in this species because they are considered to be dead end hosts (Corner *et al.*, 1981). Transmission from pigs to cattle is generally

considered unlikely because lesions generally contain few bacteria, open lung lesions are rare, and there is limited contact between cattle and feral pigs.

Prior to the national tuberculosis eradication scheme, tuberculosis caused by *M. bovis* was very common in domestic pig herds in which skim milk feeding was practised. The source of infection was the milk from those tuberculous cows which shed tubercle bacilli in their milk. The disease in pigs was controllable by removal of infected cows detected during testing. The disease was not able to sustain itself in pig herds even when the initial prevalence of infection in pigs was very high.

An investigation into a newly established feral pig population in Central Otago, (Wakelin and Churchman, 1991) revealed an overall prevalence of approximately 30% with a prevalence of 60% in two small family groups. Attention focused on feral pigs in this previously considered clean region after tuberculosis was diagnosed in cattle. It is likely that the pigs originated from endemic areas and were deliberately introduced with the aim of establishing a recreational feral population. To date, no further facts have been established to incriminate the pigs as the source of infection for the cattle in this particular incident. This region does also support a large possum population of unknown tuberculosis status. It is of particular interest that 33% of the affected pigs had lung or bronchial lymph node lesions, giving some support for respiratory transmission in this particular outbreak.

It is probable that most of the infections in feral pigs result from their scavenging of carcasses of tuberculous animals. Thomson and Challies (1988) examined the stomach contents of feral pigs shot in the Urewera Ranges and found an overall 10.6% possum content, 1.8% pig content and a smaller amount of deer content.

Historical evidence does not support theories that tuberculous feral pigs may infect cattle. It has been stated many times that the presence of tuberculous pigs has not hampered local cattle tuberculosis disease eradication programmes in Australia or New Zealand. However the possibility of occasional lateral transmission of infection from pigs into cattle should not be entirely disregarded in circumstances where prevalence in groups of pigs is very high.

Prior to the national tuberculosis eradication scheme the occurrence of tuberculosis in farmed pigs was related to the feeding of skim milk or milk from tuberculous cows. More recently,

disease occurrence in a pig herd on the West Coast (MacLaughlin, 1989) was linked with the feeding of partially cooked possums while in another incident (Nuttall, 1986) grain contaminated with excreta from tuberculous possums was thought to be responsible.

Cats

Cats, (*Felis catus)*, are susceptible to *M. bovis* infection and the probable routes of infection are ingestion of tuberculous material and via wounds due to fighting. Allen (1991) reports that between 1974 and 1991, 65 cats were diagnosed as having tuberculosis caused by *M. bovis* throughout New Zealand. Most of the cases were recorded in domestic cats. Because the disease is difficult to diagnose and is easily confused with abdominal lymphosarcomas in cats, these figures are an under-estimation of the true prevalence in domestic cats while the low numbers of feral cats reported reflect limited sampling of the feral population. Some cases have been associated with the feeding of possum meat (de Lisle *et al.*,1990; Jackson, personal observations) while the type of lesions encountered in others suggest that entry of the organism was by way of wounds associated with fighting. An outbreak of tuberculosis in cats on a North Island farm, caused by *M. bovis*, was recently reported by Orr and Thompson (1992). There were a large number of possums on this farm and the cats were occasionally fed wild deer meat. Lung, submandibular and mesenteric lesions, containing large numbers of organisms were found in these cats.

Francis (1958) reviewed 81 cases in cats which had been reported by 10 authors and concluded that the alimentary route of infection was the most important. Infected cows milk would have been an important source of infection at that time. Lesions and discharges were rich in tubercle bacilli, while lung lesions and cutaneous ulceration was not uncommon.

On the other hand, investigation of an outbreak of *M. bovis* infection in cats in a University animal house in Australia (Isaac *et al.,* 1983), gave strong indications that although the index case was probably infected by ingestion of infected meat from a knackery, in-contact cats appeared to have been subsequently infected by inhalation. An interesting finding in this outbreak was the discovery of tuberculosis in one of two brushtail possums which were housed in the same animal house. The possum had generalised lesions in lungs and liver, and *M. bovis* was isolated on culture.

Feral cats occur throughout New Zealand with densities estimated at 2 to 3 per square kilometre (Langham, 1990) to 12 per square kilometre (Fitzgerald and Veitch, 1985) on offshore islands. The population density may be expected to vary according to year round availability of food sources, the degree of competition for food from other predators and the occurrence of an often fatal disease, feline enteritis.

The use of cats as natural regulators of rabbit populations has been proposed recently and this has led to some interest in the possible role of feral cats as vectors of bovine tuberculosis. Allen (1991) cites Walker (pers. comm.) who had conducted necropsies on predators close to the Black Stilt population in the MacKenzie basin and on other animals presented to him by local people. Infection was found in 4 ferrets and 3 cats out of 1,236 rabbits, 251 possums, 109 ferrets, 70 hawks, 27 hedgehogs, 19 wallabies, 5 pukekos, 2 black-backed gulls and 41 cats. One of the 3 diseased cats was a domestic cat. The infected cats were captured on or near farms with a history of tuberculosis in farm animals.

The significance of the disease in cats remains unknown. The nature of the lesions are such that it is unlikely that a tuberculous cat could initiate infection in cattle by direct means. Ingestion of tuberculous possums is probably the method of infection of some cats and this method of spread was incriminated in an outbreak which originated in a cattery which was using possum carcasses for cat food (de Lisle *et al.*, 1990). Possums form a significant part of the diet of feral cats, (Langham, 1990; Fitzgerald and Karl, 1979), but the degree to which they prey on live possums as opposed to feeding on dead possums is unknown.

Ferrets, Stoats and Weasels

Ferrets (*Mustela furo*) are also known as fitches and polecats, although the ferret is in fact the domesticated form of the polecat. The term fitch normally refers to pelts. Ferrets belong to the Mustelid family which is also represented in New Zealand by the stoat (*Mustela erminea*) and the weasel (*Mustela nivalis*). Tuberculosis has been recorded in feral ferrets (de Lisle *et al.*, 1993; Walker *et al.*, 1993; Ragg *et al.*, 1995) and stoats (Allen, 1991; Coleman *et al.*, 1994; Ragg, 1995) and in weasels (Ryan, pers. comm.). The ferret is highly susceptible to infection with *M. bovis* (Fox, 1988; Francis, 1958). The disease progresses rapidly with the development

of lesions which contain large numbers of bacilli, indicating a poor natural resistance to the disease.

Several severe outbreaks of the disease have occurred on fitch farms in New Zealand, probably caused by the feeding of raw tuberculous possum meat. In addition, many fitch farms used feral ferrets at establishment and it is possible that infected animals were introduced at this stage. Tuberculous feral ferrets have been found in Southland, the MacKenzie Basin, at Molesworth Station in Canterbury, at Castlepoint in the Wairarapa and more recently at Otaki in the Manawatu (de Lisle *et al.*, 1993).

Ferrets are distributed throughout New Zealand, but are rare in northern Northland, Taranaki, the East Coast and Westland (Marshall, 1963). Their distribution and abundance is probably a reflection of rabbit abundance and competition for territory from feral cats, although no distribution surveys have been conducted since 1962.

Possum flesh forms part of the diet of feral ferrets but is almost certainly derived from dead animals (Fitzgerald *et al.*, 1984).

The epidemiology of the disease in this species and the question as to whether ferrets are maintenance hosts or spillover hosts is currently being examined. Ragg (1994) recorded tuberculosis prevalences of 17.2% in ferrets, 4.2% in stoats and 1.4% in cats sampled from an endemic area. She found gross mesenteric node lesions in 57% of 91 ferrets with histopathological evidence of disease. Two ferrets in this sample had discharging fistulae.

Because ferrets feed on possum carrion, they are at risk from infection if the carrion is tuberculous and may be considered as accumulators of infection at the end of the food chain. Because they are easily trapped, they may be useful as sentinels of infection in possums.

Hedgehogs and Rats

Investigations carried out in the United Kingdom and have found occasional infections in rats (*Rattus norvegicus*), but none have been found to date in New Zealand. Lugton *et al.* (in press) recently recovered *M. bovis* from five hedgehogs with gross lesions of tuberculosis in endemic regions of New Zealand and attribute the likely cause of infection to the known scavenging habits

of the animals. They point to the possible value of hedgehogs for detecting locations where tuberculous possums have died.

THE CURRENT SITUATION AND IMPLICATIONS FOR LONG TERM CONTROL

Introduction

The aim of the national tuberculosis control scheme when it was first launched in 1945 on a voluntary basis and on a compulsory basis in 1961, was eradication of the disease. Eradication at that time was considered to be a realistic objective by some, because the disease and the causative organism were thought to be largely confined to cattle. The finding of a extremely successful wildlife vector in New Zealand, capable of maintaining the disease in the absence of cattle, has effectively barred any hopes of reaching the original goal. In fact, in hindsight it may never have been a attainable target. Despite continued pressure in many countries throughout the world, the achievement of total eradication status has been variable. Factors which were relatively unimportant in transmission at the outset, such as certain management factors and presence of disease in other animals, are now recognised as important because they are not influenced by testing and slaughter policies and they are responsible for maintaining continued low levels of disease transmission.

In those countries with a residual problem, continued monitoring and vigilance are still required to maintain the disease at a very low level and prevent within herd transmission. The potential for the disease to spread is illustrated by a recent episode in Oklahoma (Schoenbaum *et al.*, 1992) when a cow was recognised to be tuberculous at slaughter inspection. The State of Oklahoma was accredited free status in 1984 and had experienced no confirmed cases since 1978. Traceback investigations showed that over 4,400 cattle in 87 herds had been exposed to infection from this particular source animal. Twelve additional cases of tuberculosis were subsequently detected but the source of infection for the first cow was not determined.

The 1992 AHB draft strategic plan recognises that total eradication is not achievable for New Zealand and has set a 5 year target for surveillance areas for reduction of the disease to a level acceptable to her trading partners, i.e. an internationally recognised Bovine Tb Free Status. This level has been defined as 0.2% of herds under movement control. O'Hara (1986) makes the point that although in the past, the prime motivation for eradication was concern for public health, it is doubtful whether the eradication approach would stand scrutiny based on cost-benefit analysis when alternatives of pasteurisation and meat hygiene afford a reasonable level of protection. Market access is clearly the main reason for pursuing a control policy in New

Zealand, and O'Hara's stance in his position as Chief Veterinary Officer in 1986, reflected the political viewpoint that Government would accept a share of the costs of control only to the extent that its share was in the public interest, as opposed to the collective interest of the agricultural industry. At that time, the industry was paying 35% of the total costs of the control programme through a levy on cattle slaughtered. Currently it contributes about 70% of budgeted AHB expenditure or 60% of total expenditure on tuberculosis control.

Implications for long term control

It is clear that the maintenance of large endemic areas containing diseased possums constitutes the main barrier to progress in control and continually threatens surveillance areas. Success in control will only be achieved when transmission of disease is interrupted throughout the cattle population. In countries which do not have a reservoir of infection, the risk of animals becoming infected diminishes at the same rate throughout the whole country, provided a test and slaughter programme is applied with equal intensity throughout the country. Under these circumstances, movement of animals from farm to farm is of low significance as a factor in maintenance of the disease, because introduced animals will carry a steadily reducing risk of disease equal to that of resident animals.

Obviously, in New Zealand, effective barriers are required to reduce the transmission from high risk areas to surveillance areas during animal trading activities, transmission from possums to cattle in endemic areas needs to be arrested, and the size of endemic areas needs to be contained or reduced at the same time,

Livingstone (1992) outlined the framework of an integrated research effort, adopted by the Animal Health Board, to be conducted in conjunction with a parallel plan for progressive containment. It has been divided into three main time frames, short, medium and long term, each having its own particular objectives but with considerable overlap and integration for projects which require continued study.

The first short term objective is to identify and elucidate those epidemiological and ecological features of the disease in possums and cattle which are of importance in maintenance and transmission of tuberculosis. An improved understanding of these factors will lead to more effective and better targeted possum control and will be used to reduce transmission from possums to cattle and deer. The data collected during these studies will be used to improve the

validity of current disease simulation models which have been designed as aids to possum and disease control. At the same time, the efficacy of recently developed tests for tuberculosis in live animals will be evaluated

The outcome of improved control strategies and policy changes emanating from these research programmes will be applied to the short term control objective of keeping the economically important surveillance areas free of tuberculosis to an internationally acceptable level. The suggested time frame for achievement of these objectives is 6 years. Progress to date in the short term studies has been encouraging and has identified some key features of the epidemiology of the disease which can be targeted in improved control strategies.

The medium term effort, which may take up to 12 years, has the objective of finding methods which may be used to reduce incidence of disease in endemic areas. The major effort during this period will be the development of vaccines for the protection of cattle and deer, and the development of more effective and acceptable poisons for possums. A complementary study will address the development of a vaccine for protection of possums against tuberculosis.

A longer term research goal of up to 16 years is aimed at development of a cost effective, humane and environmentally acceptable method of eradicating tuberculosis from possums.

Following on from the Animal Health Board strategy, the Government has since designated the control of possums and bovine tuberculosis a National Science Strategy (NSS) research area. An NSS Committee, appointed by the Ministry of Research Science and Technology, has been commissioned to "identify, coordinate and promote national priorities for possum and tuberculosis (*M. bovis*) related research in order that threats both to New Zealand's export markets and to conservation values can be eliminated".

The NSS Committee goals are similar to those set out by the AHB:

1. To identify, co-ordinate, promote and disseminate research, which will provide the information and techniques required to:
   (a) keep economically important primary production areas and animal production area and animal products free of Tb ("Stop the spread of Tb"). (Time; 1-6 years);

(b) reduce the spatial distribution and incidence of Tb in domestic and feral/wild animals ("Roll Tb back"). (Time; 3-12 years);

(c) effect a permanent and general reduction in possum numbers sufficient to ensure the sustainability of New Zealand's native plants, animals and ecosystems. (Time; 3-12 years);

(d) eliminate infected possums from New Zealand ("Wipe out possum Tb"). (Time; 6-16 years);

(e) eliminate feral/wild animal population reservoirs of Tb ("Wipe out Tb vectors"). (Time; 6-16 years).

2. To conduct research into public and Government opinion of possum/tuberculosis research and its implementation.

CHAPTER 3

A study of the topography of the lymphatic system of the Australian brushtail possum (*Trichosurus vulpecula*)[1]

[1]Submitted as Jackson Ronald, Morris Roger S. Journal of Anatomy

SUMMARY

The superficial and deep lymphatic sytems of the Australian brushtail possum (*Trichosurus vulpecula*) are described. In common with other marsupials studied to date and in contrast with most eutherians, there are no popliteal lymph nodes and efferent drainage from the inguinal lymphocentre passes directly to the deep axillary group of lymph nodes via an inguinoaxillary trunk. Efferent vessels from the tonsil pass directly to the deep cervical lymph node, which like the mandibular and parotid lymph nodes, drains independently to the superficial cervical lymph node. All subcutaneous lymph drainage passes through either the superficial cervical or the axillary lymphocentres before entering the venous system, an arrangement which is also found in kangaroos. The significance of the patterns of lesion distributions pertaining to possums with natural infections of tuberculosis caused by *Mycobacterium bovis* is discussed.

INTRODUCTION

The introduced Australian brushtail possum (*Trichosurus vulpecula*) has long been recognised as a serious pest to indigenous flora and fauna in New Zealand, yet despite vigorous control measures, possum populations have continued to increase and expand into new areas. Possums act as a reservoir for cattle for tuberculosis caused by *Mycobacterium bovis,* and this disease is endemic in several large separate regions collectively comprising about 25% of the country and containing 12 % of all cattle herds. Endemic tuberculosis in possums continues to hinder the national programme of eradication of bovine tuberculosis and threatens New Zealand's continued access to international markets for dairy, beef, and deer industry products.

In response to infection with tuberculosis, acid fast organisms (AFOs) and macrophages containing AFOs are removed from sites of infection by lymphatic capillaries and transported to regional lymph nodes for containment or destruction. Tuberculosis in possums caused by *M. bovis* is characterised by a largely ineffective host response which allows the disease to disseminate and generalise with extensive formation of granulomatous lesions in lymphoid tissues. In some species, including man and cattle, lesion distributions at initial sites of infection and regional lymph nodes have been used to determine likely routes of infection. A study of the topography of the lymphatic system of the possum was considered appropriate as a first step in the investigation of the pathogenesis of the disease and the likely routes of infection and excretion

for that species.

The investigation was designed to focus on regions of the lymphatic system of pathological and pathogenetic importance and to produce anatomical information for use in development of a systematic and comprehensive necropsy procedure for possums.

MATERIALS AND METHODS

For the study of lymphatic drainage, 28 mixed sex, independent possums were captured at night using humane cage traps baited with apple and cinnamon over a period of several weeks at locations in the Palmerston North region where possums were causing environmental damage. On the morning following capture, they were anaesthetized with intravenous pentobarbitone sodium (Nembutal 60 mg per ml, Sanofi, Boehringer Ingelheim Pty. Ltd., 50 Broughton Road, Artarmon, NSW, Australia,) following initial deep sedation with intramuscular ketamine hydrochloride (Ketamine Injection 100 mg per ml, Parnell Laboratories New Zealand Ltd., 233 Porchester Road, Takanini, New Zealand). Contrast media were introduced to selected sites and after allowing sufficient time for transport along lymphatic pathways, the animals were euthanased with intravenous sodium pentobarbitone (Pentobarb 300, 300 mg per ml, South Island Chemicals Ltd., 53 Lunns Road, Christchurch, New Zealand). Dye distribution was observed following surgical exposure of selected organs in anaesthetized animals and by use of gross dissection post mortem. Five females had young *in marsupio* (pouch young) and these immature furless animals were also used in the study. Subcutaneous lymph vessels which had been delineated using similar methods to those used for adult animals could be seen easily through the transparent skin of these furless pouch young. Some additional observations were made on drainage and location, size and number of lymph nodes at lymphocentres during approximately 500 necropsies in separate studies of prevalence of disease in other possum populations.

The dyes most commonly used were Evans Blue and Patent Blue as 5% solutions in isotonic saline, but 1% Congo Red in isotonic saline, India Ink (Hunt Manufacturing Co., Statesville, N.C. 28677, USA)and 5% carbon black in isotonic saline were also used on occasions. Congo Red was used in conjunction with either Evans Blue or Patent Blue on occasions when multiple sites were investigated and there was a need to distinguish between contrast media. This dye was found to be inferior to the blue dyes for clarity of delineation of lymphatic structures. The use

of the 5% solution of carbon black was discontinued after difficulty was experienced in detecting carbon particles in draining lymph nodes but India Ink injected directly into lymphatic vessels and lymph nodes was found to be useful for observing lymphatic vessels both in live and dead animals. The dyes were injected intradermally, subcutaneously, intramuscularly, subserosally, subconjunctivally and into the parenchyma of various organs.

Published detailed descriptions of the lymphatic systems of several species of Marsupialia were available from studies of American opossums, *Didelphys azarae* and *Didelphys marsupialis* (Azzali & Di Dio 1965), the grey kangaroo, *Macropus giganteus* (Hopwood 1980, 1988) and the koala, *Phascolarctos cinereus,* (Hanger & Heath 1991, 1994) but no reliable or published information was available for *Trichosurus vulpecula.*

Hopwood (1980, 1988) used location as the main criterion for naming lymph nodes and his terminology, based on the *Nomina Anatomica Veterinaria* (1973) (*N.A.V.*), was followed in this study.
Lymph nodes showed considerable variation in size at most locations and average sizes derived from a small sample of animals are presented here.

RESULTS

Figures 3.1(*a-b*) illustrate the supercial body regions drained by each lymphocentre. Figure 3.2 shows a diagrammatic representation of the superficial lymph nodes and the deep cervical lymphocentres of the brushtail possum and associated lymph pathways.

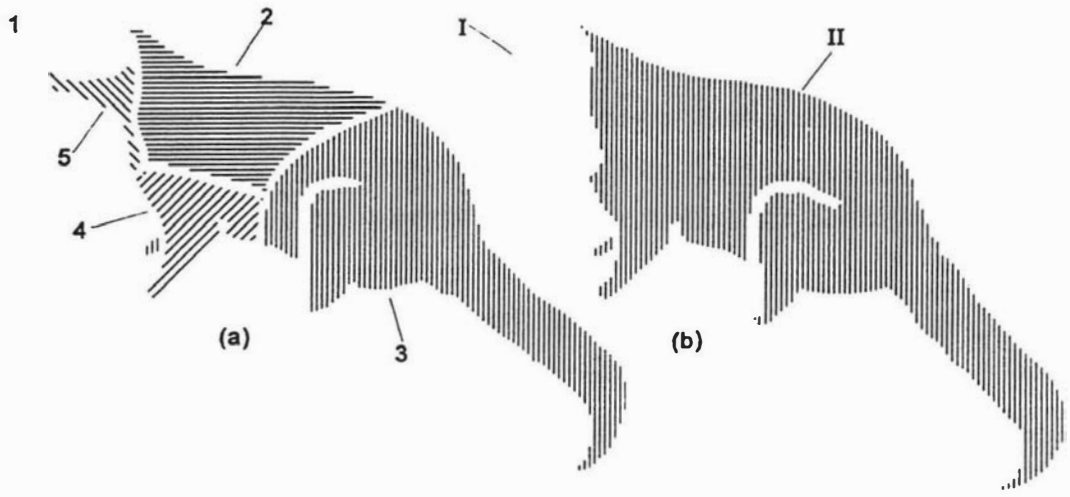

Figure 3.1(*a-b*). (*a*) Superficial body regions from which lymph drains directly to 1, parotid lymph nodes; 2, deep axillary lymph nodes; 3, inguinal lymphocentres; 4, superficial axillary lymph nodes; 5, mandibular lymph nodes. (*b*) I, Superficial body regions from which lymph drains directly or indirectly to the superficial cervical lymph nodes; II, Superficial body regions from which lymph drains directly or indirectly to the deep axillary lymph nodes.

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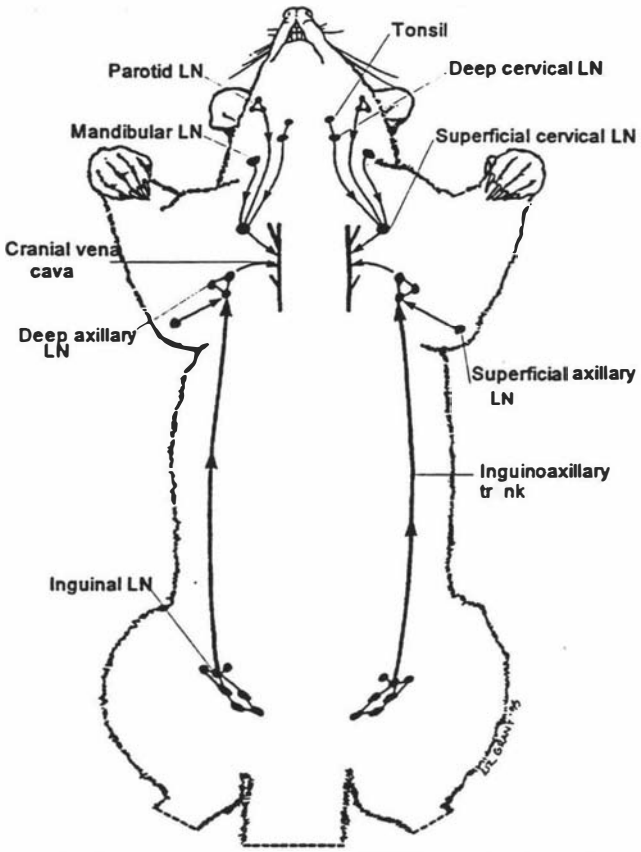


Figure 3.2. Diagrammatic representation of the superficial lymph nodes and the deep cervical lymphocentre and their efferent pathways in the brushtail possum

HEAD AND NECK

Parotid lymphocentre

Parotid lymph nodes

Location. Commonly, 2 to 3 slightly flattened spherical lymph nodes, 1 to 2 mm in diameter, were located partially or completely within the lateral part of each parotid salivary gland. The nodes were located ventral to the conchal cartilage, were closely adherent to the stroma of the salivary gland and were difficult to distinguish from gland tissue.

Afferent vessels. India Ink and Patent Blue dye were found in the parotid lymphocentre following subcutaneous and intradermal injections in the pinna, base of ear and temporal region between eye and ear. Patent Blue was found in the parotid lymphocentre following subconjunctival injection.

Efferent vessels. On each side of the neck a large efferent vessel passed caudally and diagonally across the lateral surface of the sternocephalicus muscle before passing under the muscle to the superficial cervical lymph node in its location cranial to the shoulder.

Mandibular lymphocentre

Mandibular lymph node

Location. A single oval flattened mandibular node with a long axis approximately 8 mm long was located in the space between the masseter muscle at the angle of the mandible, the mandibular salivary gland and the ventral border of the parotid gland.

Afferent vessels. Patent Blue and India Ink injected intradermally and subcutaneously into the upper and lower lips on either side of the face, and subcutaneously into the mandible and the cranioventral aspect of the neck to about level of the third cervical vertebra were traced to the mandibular lymph node on the corresponding side.

Efferent vessels. From each node, a large efferent vessel passed caudally and medial to the brachicephalicus muscle to the superficial cervical lymph node.

Deep cervical lymphocentre

Deep cervical lymph node

Location. A single ovoid deep cervical lymph node with a long axis of approximately 8 mm was located immediately caudal to the paracondylar process and was bounded laterally by the brachiocephalicus muscle and medially by the longuscolli muscle.

Afferent vessels. Patent Blue injected submucosally into the tongue and soft palate and Congo Red and India Ink injected submucosally into the ventral and dorsolateral walls of the pharynx close to the palatine tonsils were detected in the deep cervical lymph nodes. A rich submucosal plexus of vessels could be seen draining the oropharynx.

Efferent vessels. On each side, a large vessel passed along the ventral surface of the longus colli muscle to the superficial cervical lymph node.

Palatine Tonsils

Palatine tonsil

Location Palatine tonsils were firm slender and spindle shaped with a long axis approximately 4 mm long and were located submucosally and ventrolaterally in the floor of the oropharynx.

Afferent vessels No afferent vessels were demonstrated following injections of dye in the submucosa of the oropharynx.

Efferent vessels India Ink injected into the tonsils was detected in the deep cervical lymph node. An efferent vessel communicated with the deep cervical lymph node on the ipsilateral side.

Superficial cervical lymphocentres

Superficial cervical lymph node

Location A single ovoid lymph node with a long axis approximately 8 to 10 mm long occurred in a pad of fat and connective tissue cranial to the shoulder joint and clavicle and ventral border of the brachiocephalicus muscle and was bounded laterally by the omotransversarius muscle

Afferent vessels Separate afferent vessels to this lymph node were traced from the parotid, mandibular and deep cervical lymphocentres.

Efferent vessels Short efferent vessels from this lymph node entered the jugular veins caudal and close to the junctions of the internal and external jugular veins.

PECTORAL LIMB, SUPERFICIAL THORAX AND ABDOMEN

Axillary lymphocentre

This lymphocentre comprised two separate groups of nodes; a singly occurring superficial axillary node and a deep axillary group of multiple lymph nodes

Superficial axillary lymph node (N.A.V. accessory axillary)

Location This single, bilateral, slightly flattened, spherical to oval lymph node with a long axis varying from 3 to 12 mm was located subcutaneously in fat and connective tissue on the medial surface of the long head of the triceps brachii muscle at the caudolateral margin of the superficial

pectoral muscle. It could be readily palpated in most live possums, medial to the brachium.

Afferent vessels India Ink and Patent Blue were detected in the superficial axillary lymph nodes following subcutaneous and intradermal injections into the dorsum and palmar aspects of the manus, the midshaft of the antebrachium, the sternal gland region and following subcutaneous injection in the area of the ventrolateral aspect of the thorax and abdomen extending caudally from about the 6th rib to the mid abdomen.

Efferent vessels Dye passed through and from the superficial axillary lymph nodes along one or more small vessels to one or more caudally located nodes in the group of deep axillary lymph nodes.

Deep axillary lymph nodes (N.A.V. proper axillary)

Location Bilaterally, there were commonly 3 to 4 and occasionally up to 6 lymph nodes in this group One node in the group was usually much larger than the others and was irregularly ovoid and slightly flattened whereas the smaller nodes were ovoid to spherical and slightly flattened. The nodes were located in fat and connective tissue in the axillary space adjacent to the brachial blood vessels and nerves at the level of the 2nd to 4th ribs and were bounded laterally by the pectoral muscles.

Afferent vessels A large bilateral inguino-axillary vessel (inguino-axillary trunk, Hopwood, 1980) carried lymph from the inguinal lymphocentre to caudally located nodes in the group of deep axillary lymph nodes. A prominent lymphatic vessel which passed along the medial margin of the teres major to a large cranially located deep axillary lymph node collected Evans Blue dye from subcutaneous tissues in the dorsolateral neck region and dorsolateral to the scapula. A separate vessel collected dye from subcutaneous tissues in the dorsolateral aspect of the thorax and passed diagonally to lymph nodes in the deep axillary lymphocentre.

Efferent vessels The main efferent vessel from this group of nodes followed the course of blood vessels in the brachial plexus and terminated at the jugular vein immediately cranial to the first rib

PELVIC LIMB, TAIL AND MAMMARY GLAND

Inguinal lymphocentre

Inguinal lymph nodes

Location A chain up to 50 mm long and containing up to 8 irregularly shaped ovoid to circular flattened lymph nodes, was located subcutaneously along each lateral side of the inguinal region. In females with large pouch young, some of the nodes were visible through the transparent stretched skin lining the abdominal wall of the pouch.

Afferent vessels India Ink, Patent Blue and Congo Red were found in some but not all of the lymph nodes of the lymphocentre following intradermal and subcutaneous injections in the dorsal and plantar aspects of the pes. No popliteal lymph nodes were observed. India Ink and Patent Blue were found in some nodes following injections in the tail and in caudal nodes of the group following intradermal injections into the scrotum. Dye injected subcutaneously in the caudal abdominal region, the gluteal region, and the lateral aspect of the thigh passed to cranially located nodes in the group. Dye was observed in the lymphocentre on the same side of the body as injection sites in all cases except when injections were made into the substance of lactating mammary glands. Afferent vessels came from mammary gland lymph nodes located cranial to the mammary gland and along and within the chain of inguinal lymph nodes. The inguinal lymph nodes are palpable in live lean possums.

Efferent lymph vessels A large prominent occasionally doubled vessel, the inguino-axillary trunk, passed cranially along the ventrolateral aspect of the abdomen and thorax superficial to the abdominal and thoracic musculature to communicate with deep axillary lymph nodes. When delineated with dye it was visible through the skin of the pouch of females with large pouch young and showed regular constrictions, presumably indicating locations of valves, along its length.

Mammary lymph nodes

Location. Two lymph nodes were located subcutaneously cranial to each mammary gland in association with inguinal lymph nodes. The mammary lymph nodes were distinguishable from the other nodes of the inguinal lymphocentre when delineated with dye.

Efferent vessels Patent Blue injected into the substance of the mammary gland was carried to the close adjacent mammary lymph nodes.

Afferent vessels Efferent vessels entered lymph nodes in the inguinal lymphocentre and also communicated with mammary lymph nodes on the other side of the body.

ABDOMEN AND ABDOMINAL VISCERA

Iliac lymph node

Location. A single spindle shaped lymph node up to 7 mm long was located on the lateral aspect of the caudal vena cava cranial to the point where the iliac veins formed the caudal vena cava. The nodes were located between the peritoneum and the psoas minor muscle.

Afferent vessels. India Ink injected into the testicle appeared in the caudal abdominal aortic lymph nodes after passing through the internal inguinal ring in a well defined large lymph vessel. India Ink injected into the uterus also appeared in the caudal abdominal aortic lymph nodes.

Efferent vessels India Ink passed through the caudal abdominal aortic lymph nodes into a bilateral lumbar lymphatic trunk which passed cranially parallel and dorsolateral to the caudal vena cava to a cranial abdominal aortic lymph node.

Renal lymph node

Location A bilateral small ovoid flattened lymph node was located caudal to and adjacent to the renal artery.

Afferent vessels The lumbar lymphatic trunk. No injections of dye were made into kidney to determine drainage pathways from that organ.

Efferent vessels The efferent vessel continued as the lumbar lymphatic trunk which then continued cranially and dorsal to the renal vessels for a short distance prior to diverting medially to the cysterna chyli.

Colic lymph node

Location A partly lobed irregularly spherical node was located in the mesocolon close to the attachment of the root of the mesoduodenum to the dorsal abdominal wall.

Afferent vessels Patent Blue injected subserosally into the cranial descending colon transversed the mesocolon to the colic lymph node.

Efferent vessels were not demonstrated.

Cranial mesenteric lymphocentre

Cranial mesenteric lymph nodes

Location A chain of up to 20 irregularly ovoid lymph nodes were located centrally in the common mesentery along the course of the mesenteric artery. The largest nodes of the group were located at the root of the mesentery.

Afferent vessels Patent Blue injected subserosally into the jejenum, ileum and caudal part of the duodenum, passed to nodes in the chain.

Efferent vessels Patent Blue injected into the lymph nodes of the chain passed dorsally to the largest node in the root of the mesentery and from there into the cysterna chyli

Gastric lymphocentre

Gastric lymph nodes

Location A group of up to 9 spherical lymph nodes, 1 to 2 mm in diameter, were located in fat in the lesser omentum between the lesser curvature of the stomach and the pylorus. In one specimen, several spherical nodes up to 2 mm in diameter were located on the greater curvature of the stomach.

Afferent vessels Patent Blue injected subserosally into the cardia of the stomach close to the pyloric part was detected in several of the gastric lymph nodes.

Efferent vessels were not demonstrated.

Hepatic lymphocentre

Hepatic lymph nodes

Location A group of up to 4 lymph nodes were located on the mesentery extending between the hilus of the liver and the duodenum at the level of the pancreas.

Afferent vessels Patent blue injected into the substance of the liver was detected in the largest and most cranial node in the group. Patent blue injection into the subserosa of the proximal duodenum was detected in one of the hepatic lymph nodes.

Efferent vessels A short efferent vessel passed dorsally before entering the cysterna chyli.

Cysterna chyli

Location The cysterna chyli was located lateral and ventral to the abdominal aorta at a level of the first lumbar vertebra. It was in close contact with the aorta and consisted of bilateral saccules approximately 15 mm long joined by a dense plexus of communicating vessels.

Efferent vessels Following injection of India Ink into the cysterna chyli, left and right thoracic ducts were demonstrated in some specimens. In others, only a right thoracic duct was seen but it is uncertain whether this difference was real or due to the technique used. The right thoracic duct passed cranially along the dorsolateral aspect of the thoracic aorta to about the 4th thoracic vertebra where it passed to the left side before continuing through the thoracic inlet to a junction with the left jugular vein. The left thoracic duct was smaller and passed cranially to a junction with the larger duct from the other side at a level of about the third thoracic vertebra. The thoracic ducts appeared to have valves regularly distributed along their length.

Cranial mediastinal lymphocentres

Cranial mediastinal lymph nodes

Location Only 2 lymph nodes in the group of up to 8 lymph nodes, which varied in size from approximately 1 mm to 5 mm in diameter, were regularly seen and they were located to the left and right sides of the mediastinal space. The left sided node lay in the triangular space formed by the aortic arch, the brachiocephalic trunk and the trachea. In some animals, India Ink was demonstrated in this node and three more caudally located lymph nodes following cannulation of the cysterna chyli. The right sided node was located slightly caudal to the left node and was in close contact with the trachea. In some animals, dye was detected in this node following injection of Evans Blue into the parenchyma of right lung lobes. No dye was detected in any of the lymph nodes in the lymphocentre following intratracheal lavage of lung with Evans Blue or India Ink.

DISCUSSION

In Australian brushtail possums, all lymphatic drainage from subcutaneous tissues passed either through the superficial cervical or the axillary lymphocentre and the topography of subcutaneous drainage closely resembled but was not identical to that for kangaroos described by Hopwood (1980, 1988). Lymph from the inguinal lymphocentre drainage passed directly to the axillary lymphocentre via the inguino-axillary trunk and this connection was responsible for the major role of the axillary lymphocentre for drainage of subcutaneous tissues from most of the body. The inguino-axillary trunk was a feature of other marsupials studied to date, viz. American opossums, *Didelphys azarae* and *Didelphys marsupialis* (Azzali & Di Dio 1965), the grey kangaroo, *Macropus giganteus* (Hopwood 1980, 1988) and the koala, *Phascolarctos cinereus*, (Hanger & Heath 1991, 1994). A common feature of brushtail possums and all of the other marsupials was the absence of popliteal lymphocentres. Other common features were paired thoracic ducts and communications between the thoracic duct and mediastinal lymphocentre described in two Didelphids (Azzali & Di Dio 1965) and the kangaroo (Hopwood 1980). The topography of the lymphatic system of the possum and other Marsupialia so far studied shows significant differences from those of eutherians.

The pathogenesis and pathology of natural infections of tuberculosis in possums has been described by Jackson *et al.* (submitted[a,b]) and Cooke *et al.* (submitted). The pathogenesis of tuberculosis in possums in formation of granulomata in organs and draining lymph nodes appears to be similar to that in other species, although the sequence of development of lesions has not so far been determined. Tuberculosis in possums is commonly manifested as disseminated disease and it is thought that generalisation occurs rapidly once the disease starts to progress. The lymphatic system has an obvious central role in lesion formation and almost certainly has a shared role with the vascular system in dissemination of disease throughout the body.

The frequencies of tuberculous lesions of in the axillary and superficial cervical lymphocentres in infected possums sampled by Jackson *et al.* (submitted) were 0.86 and 0.29 respectively. This particular difference and the high frequencies found in the axillary and inguinal lymphocentres are difficult to explain. The authors found no physical evidence of primary site skin infection suggesting that establishment of lesions in those lymphocentres came from infected macrophages transported in the general lymphatic circulation. Their finding of statistically significantly more lesions in the left deep axillary lymphocentre than that on the right side cannot be explained by

the anatomical description presented here, although the connection between the thoracic duct and the left mediastinal lymph nodes is evidence of asymmetrical topography . In diseased animals, lymphatic pathways may be altered by obstructive lesions of tuberculosis in lymph nodes causing re-routing of flow through collateral lymph vessels and unusual patterns of establishment of lesions in draining nodes, but lesion occurrence in all other paired sites was symmetrical. Hopwood (1980) drew attention to unusual patterns of lymph flow through some lymph nodes of kangaroos but no investigation on that point was made for possums.

Lesions in mediastinal lymph nodes accompanied almost all lung lesions but the pathology seen in the mediastinal nodes was often unexpectedly mild when compared with the usual extensive and florid accompanying lung lesions. This finding and the relatively small size of the mediastinal nodes indicates that possums may possess peculiar lung clearance mechanisms.

This study was carried out as a first step in providing a better understanding of the epidemiology and pathogenesis of naturally occurring tuberculosis in possums. Further studies involving both normal and diseased animals are warranted to clarify unresolved issues. The description presented here is incomplete and several areas of gross anatomy, including drainage from lung, the function of mediastinal lymph nodes and the unexplained high prevalence of lesions in the deep axillary lymphocentre are worthy of further more detailed investigation. In particular, an elucidation of mechanisms of clearance of bacteria from lungs, the role of macrophages, the means and paths by which macrophages are transported, and the filtering efficacy of lymph nodes, would improve the understanding of the pathogenesis of tuberculosis in the possum.

ACKNOWLEDGEMENTS

The professional advice and encouragement from Dr. Alex Davies is gratefully acknowledged. The figures were drawn by Liz Grant of the Ecology Department at Massey University.

CHAPTER 4

A study of environmental survival of *Mycobacterium bovis* in selected locations in New Zealand[1]

[1]Submitted as: Jackson R, Morris RS, de Lisle GW. New Zealand Veterinary Journal. Several tables have been included here for more complete reporting.

ABSTRACT

Mycobacterium bovis organisms absorbed on cotton ribbons were placed in different natural habitats in New Zealand. *Mycobacterium bovis* was not re-isolated from ribbons placed on pasture after 4 days. Survival on ribbons was longest in brushtail possum dens and the maximum period of survival in dens was less than 7 days in summer and greater than 14 days but less than 28 days in winter and spring. The maximum period of survival on a forest floor was intermediate between pasture and dens and was less than 4 days in summer and greater than 14 days but less than 28 days in winter. The overall probability of survival was influenced by season and was shortest in summer and longest in spring and winter. Survival time increased as minimum daily temperatures decreased. These studies showed there was a relatively short period of survival of *M. bovis* outside hosts and support a conclusion that environmental contamination of pasture, particularly in summer months, may be relatively unimportant in the epidemiology of tuberculosis in cattle, deer and possums.

INTRODUCTION

Tuberculosis in cattle in New Zealand caused by *Mycobacterium bovis* is complicated by the presence of the disease in the Australian brushtail possum, *Trichosurus vulpecula*, which acts as a wildlife reservoir and vector of the disease. This introduced arboreal folivore has successfully colonised most of New Zealand and bovine tuberculosis is currently endemic in the species in five regions which collectively comprise about 20% of the country. The overall prevalence is approximately 0.01–0.03, but the disease exhibits marked spatial clustering, with prevalences commonly varying between 0.2 and 0.3 in foci of infected possums. Conventional control methods aimed at eradication of the disease in cattle and possums have been only partly effective and the total size of the endemic areas has increased over the past 20 years.

Although Pfeiffer (1994) produced evidence supporting a conclusion that *M. bovis* is transmitted directly from mother to offspring during the prolonged rearing period exhibited by marsupials, this mechanism alone is insufficient to produce persistence of the infection in possum populations. Additional direct and/or indirect transmission mechanisms are thought to be necessary. The contribution which indirect transmission may make is unclear, although possum behaviour in the wild indicates that the most likely opportunities for indirect transmission occur

during grooming, concurrent and sequential den-sharing, territorial marking activities and during grazing on shared pastures.

There is now strong evidence that direct transmission of *M. bovis* from possums to cattle and deer occurs mainly during close contact between terminally ill possums and cattle and deer (Paterson, 1993; Paterson and Morris, 1995; Sauter and Morris, 1995). However, contaminated habitats may provide opportunities for indirect transmission.

The ability of *M. bovis* to survive outside its hosts for prolonged periods of time under favourable conditions has been demonstrated in observational studies following both natural and artificial contamination of various environmental sites, including cattle faeces (Stenhouse Williams and Hoy, 1930; Maddock, 1933; 1934), on pasture (Maddock, 1933; 1934; 1936; Reuss, 1955;Schellner, 1956; 1959), in soil (Maddock, 1933; 1934), and on the body surface and in the intestines of *Musca domestica* (Saad, 1964). Naturally infected and experimentally contaminated faeces were used in studies on the persistence of *M. bovis* in faeces and on pasture (Maddock, 1936; Reuss, 1955). Other pasture studies conducted by Schellner (1956; 1959) and Maddock (1933; 1934) employed irrigation of grazing plots with suspensions of tuberculous organs of cattle, and overall the studies indicated that the duration of infectivity for cattle was much shorter than the period for which organisms could be recovered artificially. The high levels of contamination used in those early studies were considered relevant to those eras when generalised and advanced cases of tuberculosis were common in cattle. Although they provide useful information, those studies do not adequately represent currently encountered environmental conditions in New Zealand, which are usually associated with a low prevalence of tuberculosis and early disease states in cattle.

Other studies of persistence of *M. bovis* include those of Genov (1956) on the effects of temperature and sunlight on cultures mixed with various substrates in a variety of locations, Donsel and Larkin (1977) on survival of *M. bovis* BCG mixed with sewage effluent and sludge sprayed on to vegetable crops, Duffield and Young (1985) on survival in soil in tropical Australia, and a series of environmental studies specifically designed to clarify aspects of the epidemiology of tuberculosis in badgers (Anon, 1979; Little *et al.*, 1982).

The contributions which these studies have made to an understanding of the relative importance of persistence of *M. bovis* outside of the host in the epidemiology of M. bovis infections have recently been reviewed by Morris *et al.* (1994).

During a series of integrated studies of the epidemiology of tuberculosis in possums which included simulation modelling of the disease (Pfeiffer, 1994), it became apparent that more precise information was needed about survival of *M. bovis* organisms in possum dens, and at pasture and forest floor locations where possums interact among themselves, and with cattle, and with other domestic and wild animal species.

The studies described in this paper were designed to investigate the survival of *M. bovis* organisms in possum dens, on the ground under forest and scrub canopy, and on open pasture. Studies were carried out at the study site described by Pfeiffer and Morris (1991) located at Castlepoint which is in the Wairarapa region of New Zealand.

MATERIALS AND METHODS

Substrate material preparation

Strips of cotton ribbon (Stabilo-Ohpen 196, Superfine Wasserfest) about 50 cm long, were marked with ten permanent marks at 3 cm intervals. The strips were autoclaved and dried prior to placement of 50 µl of an inoculum of *M. bovis* on each 3 cm segment. All studies were conducted using the DNA restriction endonuclease type of *M. bovis* most commonly isolated from tuberculous possums during the Castlepoint study. The inoculum was prepared by growing *M. bovis* to late log phase in Tween albumin broth (TAB; Dubos broth base, Difco Laboratories, supplemented with 0.006% v/v alkalinised oleic acid, 0.5% w/v albumin fraction V and 0.25% glucose). The concentrations of the inocula were approximately 1–5 x 10^8 organisms per ml. The identity of the cultures was verified by determining the sensitivity to 10 µg/ml isoniazid and 10 µg/ml thiophene-2-carboxyhydrazide. The purity of the *M. bovis* culture was checked by inoculating a blood agar plate and incubating at 37 ^{0}C for 3 days to late log phase.

The ribbons were chilled and transported to the study area in an insulated light-proof container. Duplicate ribbons, with the free ends in contact with the ground cover, were attached about 12 cm above ground level to stainless steel pegs inserted into the ground about one metre apart at each of three separate open pasture and three separate forest floor locations at the study site. Duplicate ribbons were also attached to both ends of stainless steel rods and placed in three separate dens located in flax (*Phormium tenax)* bushes, the openings of which were then closed with a wide mesh stainless steel grid to prevent entry of possums. The forest floor locations were under a large gorse (*Ulex eurorpaeus*) bush and under mixed manuka (*Leptospermum scoparium*) and mingimingi (*Leucopogon fasciculatus*) scrub. Controls were kept protected from light at room temperature and at 5° C in the laboratory and also within and outside weather recording boxes.

Timing of studies, test material collection and subsequent bacteriological examinations

Four separate studies using the same methodology were carried out, coinciding with the start of each climate season. Studies commenced on 27 February 1992 (autumn), 6 June 1992 (winter), 9 September 1992 (spring) and 9 January 1993 (summer). In the autumn study, the first marked section of ribbon was removed after 4 days at pasture locations and after 7, 14, and 28 days at other locations. In subsequent studies, times to the first test on forest floor and inside den locations were reduced to 4 days and times to the first test on pasture were reduced to 2 days for

the spring and summer studies. Sterile instruments were used when removing test material which was placed in sterile plastic containers and transported promptly in a chilled state in insulated containers to the Central Veterinary Laboratory at Wallaceville. Although the use of prescribed data collection times adds to data imprecision in survival studies, they were necessary because of logistical and financial constraints associated with collection of samples and laboratory culture.

Bacteriology

Each 3 cm strip was placed in 5 ml of TAB, vigorously vortexed for 30 seconds and 100 µl was inoculated on to a plate of modified 7H11 agar (Mycobacteria 7H11 agar, Difco Laboratories, Detroit, Michigan USA, containing 10% sheep serum, 0.25% lysed sheep red cells, ticarcillin disodium 100 µl/ml, polymixin B 200 IU/ml, amphotericin B 10 µg/ml and trimethoprim 10 µg/ml). Some of the bacterial suspensions were diluted in TAB and inoculated on to plates to determine the number of organisms which could be recovered from the strips. Plates were incubated in sealed plastic bags at 37 ^{o}C for 2 months. The number of colonies of *M. bovis* were counted and for each strip from which *M. bovis* was isolated, the isolate identified as *M. bovis* using acid-fast staining and microscopy, and the use of a monoclonal antibody to MPB70 in an immuno-peroxidase test.

Weather data recording

Weather data loggers (Squirrel memory loggers, MQW32, Grant Instruments Ltd., Barrington Cambridge, England) were installed at three locations on the study site. At a forest floor and two pasture sites, temperature and relative humidity were recorded hourly by humidity probes (Vaisala, VH-L-Z1), suspended about 12 cm above ground level within specially constructed weather recording boxes. The boxes were made of light galvanised sheet metal, painted white, and were rectangular in shape with a pitched roof, wide overhanging eaves and metal louvres along both long sides. The weather recording boxes were 80 cm long, 35 cm wide and the apex of the roof was 45 cm high.

At one pasture site, a temperature recording probe (Type CM, Grant Instruments Ltd., Barrington Cambridge, England) was located in an adjacent den. At the other pasture site and the forest floor site, a Vaisala humidity recording probe was suspended about 12 cm above the floor of an adjacent den. Data were downloaded from the data loggers to a portable IBM compatible

computer using the Eltek Control/Transfer program (Eltek Ltd. 35 Barton Rd, Haslingfield, Cambridge, England).

Data were stored in Paradox for Windows Version 4.5 and Quattro Pro for Windows Version 5 (Borland International). Unless designated otherwise, analyses were conducted in Statistica for Windows (Statsoft Inc., 2325 East 13th Street, Tulsa, USA). The degrees of freedom for χ^2 tests are reported as subscripts after χ. Cox's proportional hazards regression procedure in Number Cruncher Statistical System, Version 5.03, (Dr. Jerry L. Hintze, Kaysville, Utah, USA) was used to study the effects of other factors on survival time using 4-day first test result data. Hourly temperature and relative humidity data were summarised into daily means, maxima and minima and standard deviations were calculated for both temperature and relative humidity for each location during winter, spring and summer studies. All summary daily temperature and relative humidity statistics, number of organisms per section of ribbon for each season, and location and season (each transformed to dummy variables), were included as covariates in the regression analysis.

RESULTS

Culture results

The number of organisms recovered from ribbons following inoculation and prior to placing them in field positions were 3.5 x 10^5/section of ribbon for the autumn study, 7.1 x 10^5 for winter, 1.9 x 10^6 for spring and 8.8 x 10^6 for summer. Summary data presented in Table 4.1 show the numbers of positive, negative and contaminated results of culture tests from pasture, forest floor and dens during the test periods in autumn, winter, spring and summer.

Table 4.1. Numbers of positive, negative and contaminated culture test results from ribbons replicated at each of 3 sites on pasture, a forest floor and in dens and number of samples not tested

Culture results	autumn 1992				winter 1992				spring 1992				summer 1993			
Pasture sites	+	-	c	nt	+	-	c	nt	+	-	c	nt	+	-	c	nt
2 days				6				6	1	5			1	5		
4 days		6				6				6				6		
7 days		3	3			5	1			6				6		
14 days		6				6				6				6		
28 days		6				6				6				6		
Forest floor																
2 days				6				6				6				6
4 days				6	3	2	1		4	1		1		6		
7 days		4	2		2	4			1	4		1		6		
14 days		6			1	5				5		1		6		
28 days		6				6				5		1		6		
Dens																
2 days				6				6				6				6
4 days				6	6				6				6			
7 days	4	2			1	2	3		5	1				6		
14 days		6			4	2			4	2				6		
28 days		6				6				6				6		

+ = *M. bovis* cultured; - = *M.bovis* not cultured; c = contaminated; nt = not tested

In the design of the study, test material collection times were prescribed as 4 days for pasture locations and 7, 14, and 28 days, and 3, 6, 9 and 12 months for all other locations, with at least

one negative culture test result at a collection time subsequent to the last positive culture test required as evidence of nil survival at a particular time.

The time periods to the first test for the three locations were "best guess" estimates derived from literature reviews of previous studies and knowledge of the New Zealand environment. In the autumn study, first tests were made at 4 days for pasture locations and 7 days for forest floor locations, but culture results at those times were negative, indicating that the interval between the start of the trial and the first tests was too long. The altered time period to the first test, introduced subsequent to autumn 1992, improved the accuracy of results for the subsequent tests but introduced problems for data analyses which ideally required time periods common to all studies.

In order to accommodate those problems, analyses were conducted using both common first time periods for all seasons and common reduced first time periods for those studies in which suitable data was collected. For survival analyses, the time of death of organisms on each ribbon was calculated as the mean of the last time a culture test was positive and the first time a culture test was negative. Cases where a contaminated result was recorded between a positive and a negative result were dropped from the analyses.

Survival probabilities of *M. bovis* organisms on pasture, a forest floor and in dens over all seasons.

Figure 4.1 illustrates the survival probability curves of *M. bovis* organisms on pasture, on a forest floor and in dens using aggregated four-season data and 7-day first test results. The Log Rank statistics set out in Table 4.2 were associated with highly significant differences ($\chi_2^2 = 21.39$, $p < 0.0001$) for between group probabilities of survival at these locations.

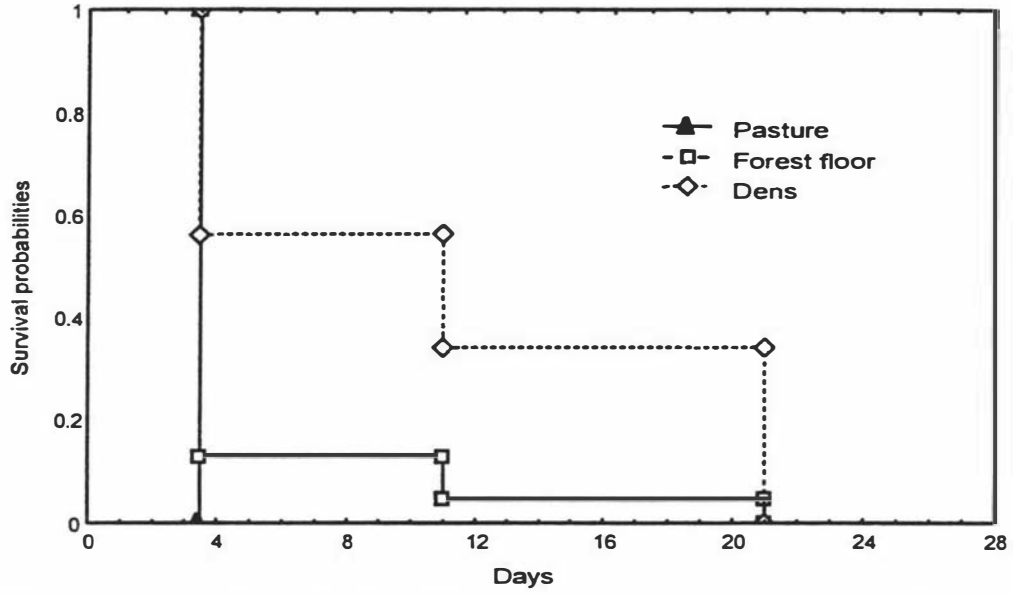


Figure 4.1. Survival probabilities of *M. bovis* organisms on pasture, forest floor and in dens, calculated using aggregated data from spring, summer autumn and winter, with 7-day test results as first measurements

Table 4.2. Group medians, means and Log Rank statistics from comparison of survival probabilities of *M. bovis* organisms on pasture, forest floor and in dens over four seasons calculated using 7-day test results as first measurements

Group	N	Group median	Group mean	Log Rank Sum	Log Rank Mean
Pasture	28	3.5	3.5	6.137	0.219
Forest floor	22	3.5	4.9	2.509	0.114
Dens	23	11	11.1	-8.646	-0.376

N = sample size

Statistical significance was retained ($\chi_2^2 = 25.97$, $p < 0.0001$) in the Log Rank (Lee, 1980) statistics set out in Table 4.3, when the more precise 4-day first measurement data were used. Figure 4.2 illustrates the survival probability curves of *M. bovis* organisms on pasture, forest floor and in dens using aggregated winter, spring and summer data and 4-day test results. The curves show that *M. bovis* died most rapidly at pasture locations, with almost all dead by 2 days and survival was limited to only one ribbon (3 colony forming units (cfus)) at 4 days. Mortality rates were also high during this 4-day period for organisms located on the forest floor and were intermediate between rates for pasture and for dens.

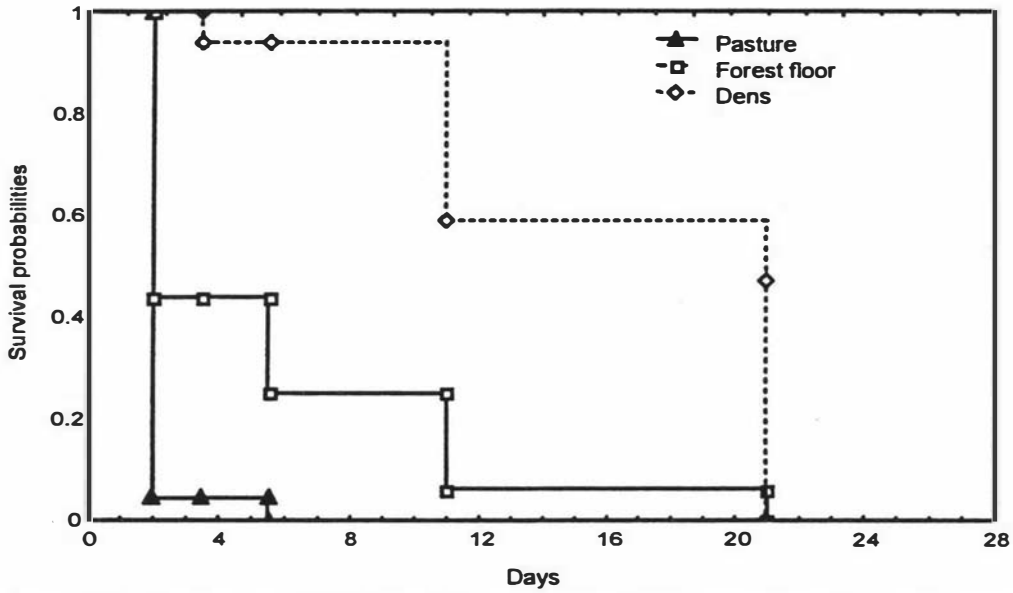


Figure 4.2. Survival probabilities of *M. bovis* organisms on pasture, forest floor and in dens during spring, summer and winter, calculated using 4-day test results as first measurements.

Table 4.3. Group medians, means and Log Rank statistics from comparison of survival probabilities of *M. bovis* organisms on pasture, forest floor and in dens during spring summer and winter using 4-day test results as first measurements

Group	N	Group median	Group mean	Log Rank Sum	Log Rank Mean
Pasture	23	2	3.5	9.559	0.416
Forest floor	16	2	4.9	1.108	0.069
Dens	16	10.5	11.1	-10.668	-0.667

N = sample size

Association between seasons and survival of *M. bovis* organisms on pasture, forest floor and den locations

Survival on pasture

No significant difference was demonstrated between survival on pasture in spring and summer (Log Rank $\chi_2^2 = 0.057$, p = .97) when survival probability curves were constructed using first test results recorded at two days (spring n = 6 and summer n = 7).

Survival probability curves for pasture during spring, summer, autumn and winter were also constructed using first test results recorded at 4 days (autumn n = 6, winter n = 8, spring n = 8 and summer n = 7). No statistically significant seasonal differences were demonstrated (Log Rank $\chi_3^2 = 3.143$, p = 0.37).

Survival on a forest floor

Survival probabilities for forest floor locations during spring, summer autumn and winter were calculated using first results recorded at seven days (autumn n = 6, winter n = 6, spring n = 5 and summer n = 5). No statistically significant between season differences were demonstrated (Log Rank $\chi_3^2 = 3.79$, p = 0.29 for an aggregated four-season comparison), and no significant differences were found between any two-season combinations.

Survival curves for forest floor locations during spring, summer and winter were constructed using first test results recorded at 4 days (N winter = 5, spring n = 6 and summer n = 5). The overall three season comparison test was not significant ($\chi_2^2 = 3.58$, p = 0.17), but a difference (p = 0.06) was recorded between spring and summer survivals. The analysis suggested a difference (p = 0.1) between winter and summer survival on a forest floor when the more precise 4-day first test result data were used, but no differences in survival were shown between winter and spring. The survival probability curves for comparisons of seasonal survival on forest floor using 4-day first test result data are illustrated in Figure 4.3.

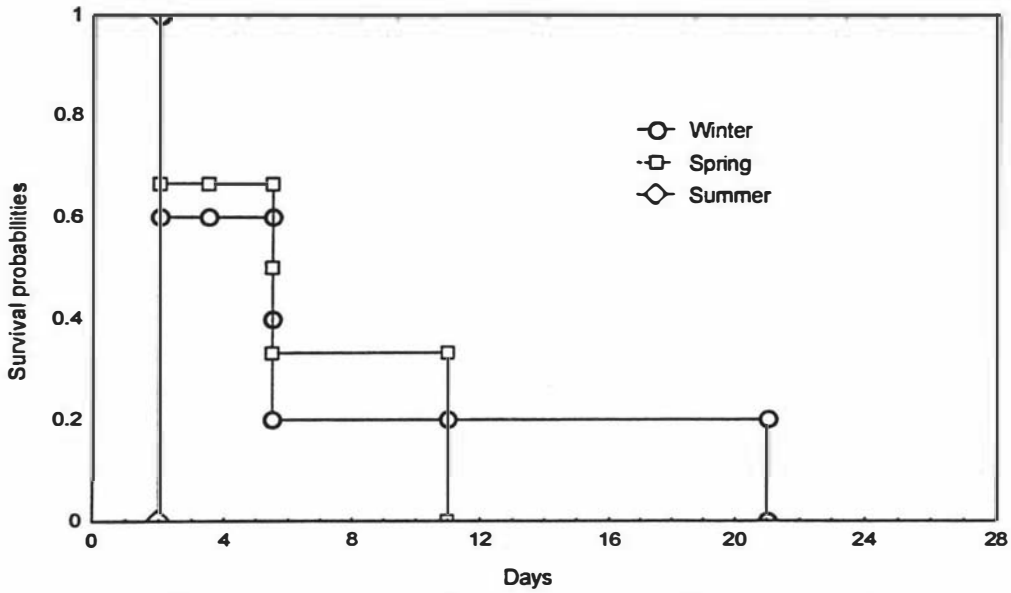


Figure 4.3. Survival probabilities of *M.bovis* organisms on forest floor during winter, spring and summer calculated using 4-day test results as first measurements.

Survival in dens

Significant seasonal differences were demonstrated (Log Rank $\chi_3^2 = 12.67$, $p = 0.005$) when survival probabilities for *M. bovis* organisms in dens during spring, summer, autumn and winter were calculated using first test results recorded at seven days (autumn n = 6, winter n = 5, spring n = 6 and summer n = 6).

Survival curves for *M. bovis* in dens were then compared between all two-season combinations using the same 7 day first test result data. The Log Rank statistics for the tests of all two-season combinations are set out in Table 4.4. All except those between spring and winter showed statistically significant differences. The survival probability curves for comparisons of seasonal survival in dens using 7-day first measurement data are illustrated in Figure 4.4.

Table 4.4. Log Rank statistics from comparisons of between season survival probabilities of *M. bovis* organisms in dens using 7-day test results as first measurements

Seasons	Log Rank statistic	Variance	Z - value	p - value
Spring - Summer	-2.5	1.014	-2.48	0.01
Spring - Winter	-0.35	0.689	-0.43	0.7
Spring - Autumn	2.28	1.220	2.06	0.04
Summer - Winter	2.18	0.694	-2.62	0.01
Summer - Autumn	-2.0	0.727	-2.35	0.02
Autumn - Winter	2.36	1.140	2.21	0.03

Survival probabilities for organisms in dens during spring, summer and winter were also calculated using the more precise first test result data which were taken at 4 days (N winter = 5, spring n = 6 and summer n = 6). Significant overall seasonal differences were demonstrated (Log Rank $\chi_2^2 = 7.56$, p =0.02).

Survival curves for *M. bovis* in dens were compared between seasons using the same 4-day first measurement data. The Log Rank statistics for the tests between seasons are set out in Table 4.5 and show similar results to those produced using 7-day first test results. Between two season comparisons again showed significant differences with the exception of winter and spring. The survival probability curves for comparisons of seasonal survival in dens using 4-day first test result data are illustrated in Figure 4.5.

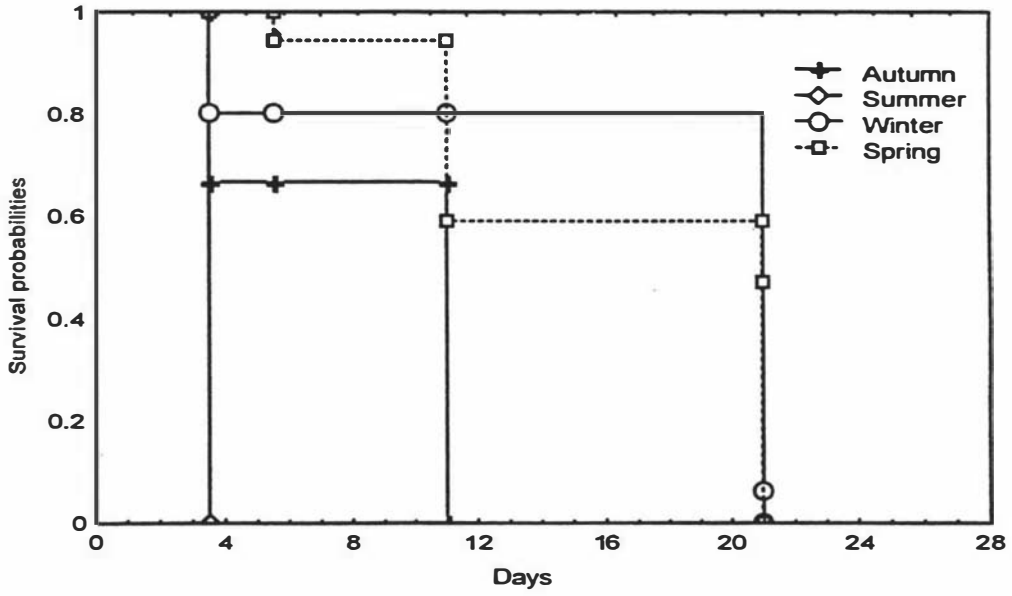


Figure 4.4. Survival probabilities of *M. bovis* organisms in dens during autumn, winter, spring and summer calculated using 7-day test results as first measurement data..

Table 4.5. Log Rank statistics from comparisons of between season survival probabilities of *M. bovis* organisms in dens calculated using 4-day test results as first measurements

Seasons	Log Rank statistic	Variance	Z - value	p - value
Spring - Summer	-3.000	1.182	-2.760	0.006
Spring - Winter	-0.254	0.684	-0.308	0.76
Winter -Summer	-1.854	0.902	-1.952	0.05

Location and weather effects

The final regression model selected, in which SUMMER and DENS were used as referent variables, is set out in Table 4.6. D is an index value similar to R^2 in regression representing the relative influence of the variable. Antilogs of the Beta statistics were calculated to give another indication of the size of the effect of significant variables compared to the referent variables. Daily minimum temperature (DAYMINT) was statistically significant and was included in the final model. Figure 4.6 illustrates the daily minimum temperatures recorded during the study at different locations and the daily mean temperatures on pasture for winter, spring and summer. The similarities of the temperatures experienced in spring and winter are noteworthy.

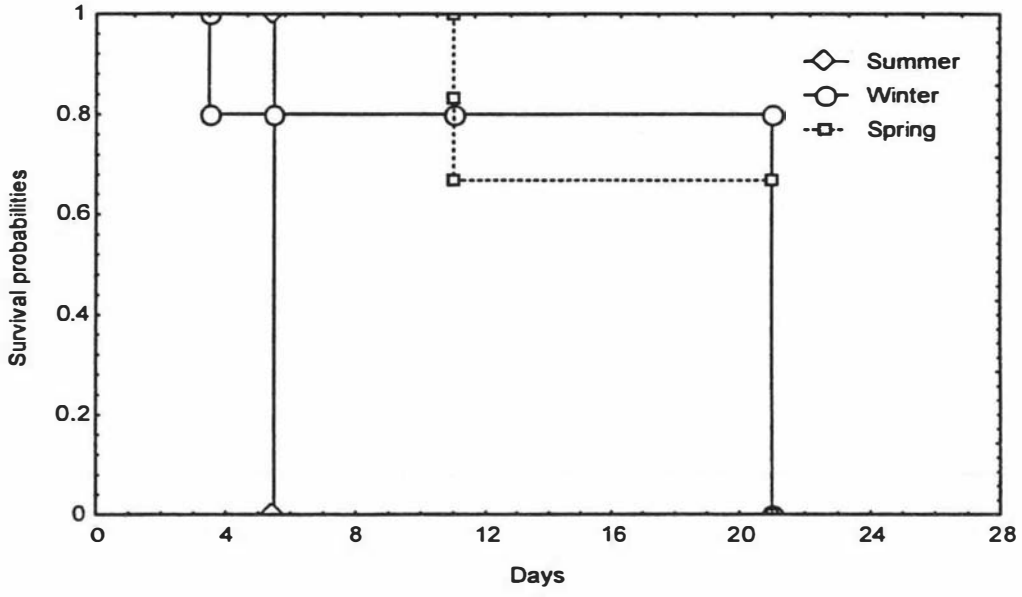
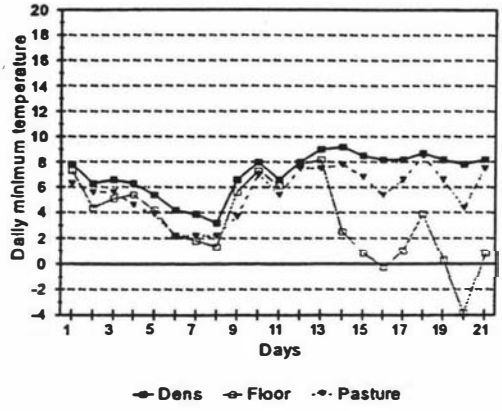


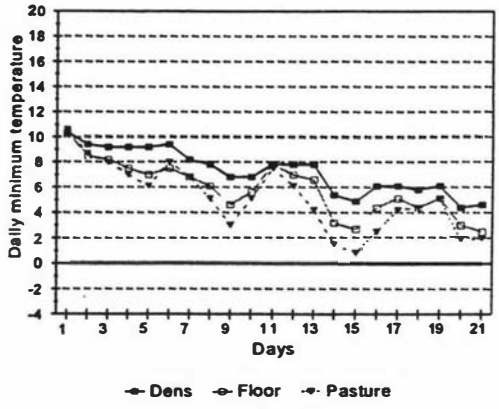
Figure 4.5. Survival probabilities of *M. bovis* organisms in dens during winter, spring and summer calculated using 4-day test results as first measurement data.

Table 4.6. Cox's proportional hazard regression model for survival of *M. bovis*

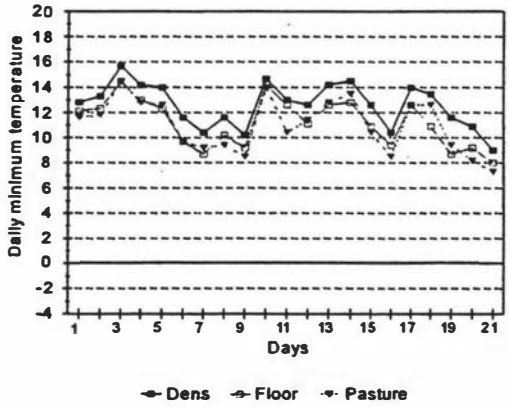
Variable	p - value	D	Beta	Exp^{Beta}
PASTURE	<.0001	0.257	1.922	6.8
FLOOR	.002	0.154	1.244	3.4
DAYMINT	.008	0.118	0.147	1.15



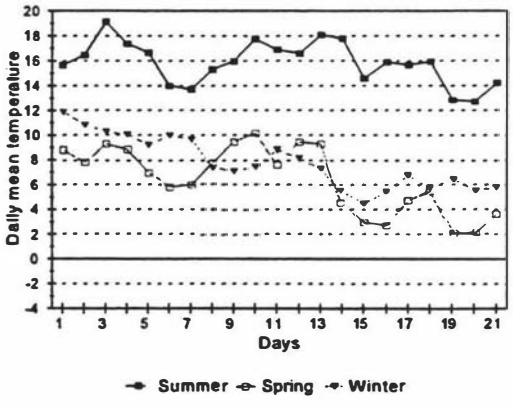
Spring (6a)



Winter (6b)



Summer (6c)



(6d)

Figure 4.6. Daily minimum temperatures on pasture, a forest floor and in dens during spring (6a), summer (6c) and winter (6b) and daily mean temperatures at the study site at those times (6d).

DISCUSSION

In these studies, the survival of *M. bovis* on sample ribbons was measured at predetermined intervals. The results need to be interpreted cautiously with due regard to the differences which exist between the true state of nature and the experimental conditions necessarily applied in the studies. A number of strategies were used to minimise these differences and to mimic the state of nature as closely as possible. Discussion about the methodology which was used is included here because the results only allow deductive explanations to be made and need to be interpreted intuitively when applied to field situations.

Studies using natural locations at which a 5-year study of the natural disease was being conducted were selected in preference to laboratory bench or climate house studies in order to approximate natural conditions more closely. Cotton ribbons were used as a substrate for inocula because of their natural plant material composition and an expected response to weather and solar radiant energy similar to that for natural vegetation. The number of organisms used in this study ($3.5 - 16.0 \times 10^5$ / 3 cm strip) was considerably less than the 5×10^8 / ml recorded by Smith[20] in exudate from a discharging lesion in a possum and often less than that used in comparable survival studies. Furthermore, the presence of organic material such as inflammatory exudate may offer some protection against potentially lethal environmental factors.

Ribbons were cultured without a decontamination step and this strategy was justified by the low number of contaminated cultures (13 contaminated out of 369 cultures). The decision to dispense with decontamination was taken after a preliminary study indicated that about 90% of *M. bovis* present was killed when subjected to a mild sulphuric acid decontamination.

Working from general principles, Wray (1975) and Kelly and Collins (1978) suggested temperature, moisture, exposure to the desiccating effects of sunlight, and ultraviolet light as factors which might influence the survival of *M. bovis* on pasture. The effects from all of these variables could not be investigated, but temperature and relative humidity recordings were made to try to identify important weather parameters which could be associated with survival. For this reason, the multivariate Cox's proportional hazards regression model does not fully describe the extent and nature of physical effects on survival. Rain may have influenced subsequent organism recovery rates if it caused organisms to be washed off ribbons. Any such confounding effect from rain should have been greatest on pasture, intermediate on forest floor and least in dens,

reflecting the different shelter in the three sites. Rainfall was not measured at the location of the strips but relative humidity recordings indicate that some rain fell during the winter and spring studies. No rain fell during the first 7 days of the summer study, although by that time, all ribbons in all locations were culture negative.

Despite these limitatiions, this analysis clearly showed that survival time was shortest on pasture, intermediate on forest floor and longest in dens and, that as mean daily temperatures declined, the period of survival increased. This gradation according to location was not unexpected, although overall survival times were shorter than anticipated. Ribbons in dens were not exposed to direct light, were protected from rain and were not subjected to the wide daily fluctuations in temperature and relative humidity experienced in more exposed sites. The degree of protection from adverse weather on forest floor was less than in dens but greater than on pasture.

No significant between-season differences were shown for survival on pasture but few organisms survived in any season. Additional testing at more frequent intervals would be required if more precise information was required for that time period.

A between-season difference ($p = 0.06$) was recorded between spring and summer on the forest floor and there was a suggestion of a difference ($p = 0.1$) between winter and summer at the same location. Between season differences were also apparent for survival in dens, with the exception of between winter and spring. This was probably a reflection of the similar weather patterns encountered in those seasons.

The proportional hazards regression model reinforced the findings from the between group analyses. The finding of a significant effect related to daily minimum temperatures was not unexpected, although a relationship between the daily variability of weather statistics, and in particular temperature, was also expected because of the more equable range of temperatures recorded in the well sheltered and insulated dens. While in the laboratory control organisms survived well at 5^0 C compared to those at room temperature, such continuous low temperatures would never occur in the natural habitat.

The central issue of epidemiological interest is not only how long *M. bovis* survives, but how long surviving organisms remain infective (Morris *et al.*, 1994). Organisms may persist for very long periods in protected and sheltered locations such as in soil, but by virtue of their location be either

inaccessible to a host or only available for infection by an inefficient route. Large doses of organisms are required to successfully infect cattle by the alimentary route (M'Fadyean 1910) and in that species environmental contamination other than by aerosol is relatively unimportant for initiating infection. Experimental infections of cattle have been produced following contamination of pasture with emulsions of *M. bovis* organisms, produced from macerations of tuberculous organs of cattle (Maddock, 1934; Schellner, 1956), but the contamination rates used were very high. Schellner (1959) employed a suspension containing 10^5 to 10^7 organisms per ml applied to pasture at a rate of 1 litre per m^2. The weight of evidence from epidemiological and pathological investigations favours the respiratory route as the main route of infection in cattle (Francis, 1947; 1958; 1971; Stamp, 1948; Collins and Grange, 1983; McIlroy *et al.*, 1986; Neill et al., 1988; Pritchard, 1988). The relative efficiencies and importance of available routes for deer and possums are less certain, although there are indications from pathogenesis studies in possums (Jackson et al., 1995a; 1995b) and behavioural studies in deer cattle and possums (Paterson, 1993; Paterson and Morris, 1995; Sauter and Morris, 1995), that the respiratory route is the major route in those species. As with cattle, environmental contamination apart from aerosols may not be important for initiating infection. During a 5-year longitudinal study at Castlepoint, there was no evidence to suggest that the risk of tuberculosis for possums which were caught in box traps occupied by a clinically tuberculous possum within the previous 4 days was increased (unpublished observation).

Our studies of survival showed there was a relatively short period of survival of organisms outside hosts and support a conclusion that environmental contamination of pasture, particularly in summer months, may be relatively unimportant in the epidemiology of tuberculosis in cattle, deer and possums.

ACKNOWLEDGMENTS

The assistance of C. Sauter and D.U. Pfeiffer in the translation of several papers from German to English, and the friendly co-operation and assistance of R. Goile, D. Lewis and W. Maunsell at the farmland site are gratefully acknowledged. We thank the support staff at the Central Veterinary Laboratory at Wallaceville for their processing of samples. Financial support was provided by the Animal Health Board.

CHAPTER 5

Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichosurus vulpecula*): I. An epidemiological analysis of lesion distribution.[1]

[1] Submitted as Jackson R, Cooke MM, Coleman JD, Morris RS. New Zealand Veterinary Journal

ABSTRACT

Gross and microscopic lesion distributions and culture test results are described for 73 tuberculous possums recovered from a series of cross-sectional studies involving approximately 500 detailed necropsies. Pathological findings from 11 terminally ill tuberculous possums are also described. Quantitative epidemiological techniques have been applied to lesion site data to assess factors influencing the pathogenesis of the disease. In possums with gross lesions, the number of distinct body sites affected varied from one to 10 per animal, with a mean of 4.6. The total number of gross plus microscopic lesions varied from one to 28 per animal with a mean of 11.6, indicating that the degree of generalisation of disease was much greater than appeared grossly. Of 119 possums with no gross lesions which were subjected to additional examinations, tuberculosis was diagnosed in ten (8.4%) by histology or culture of pooled lymph nodes. Among cross-sectional sample tuberculous possums, lesions were found in lungs in 85%, in axillary lymphocentres in 85%, in inguinal lymphocentres in 69%, and in either axillary or inguinal lymphocentres in 95%, indicating that the disease spread rapidly to multiple body sites. Proportionately more males than females were infected (Relative Risk = 1.78). When cross-sectionally sampled infected and non-infected possums were compared, no significant associations were found between the presence/absence of disease and either age or indices of body condition, although debility was seen in animals with terminal illness.

INTRODUCTION

There is an incomplete understanding of the pathogenesis of tuberculosis in brushtail possums (*Trichosurus vulpecula)* caused by *Mycobacterium bovis*. Present knowledge has come from results of experimental infections (Bolliger and Bolliger, 1948; Corner and Presidente, 1980; 1981; O'Hara *et al.*, 1976; Buddle *et al.*, 1994; Pfeffer *et al.*, 1994), from examinations of possums which have died from tuberculosis during the Castlepoint longitudinal study (Pfeiffer and Morris, 1991) and from post mortem surveys (Julian, 1981; Coleman, 1988; Anon, 1975; Anon, 1986).

Generalised and rapidly progressive fatal disease has been shown to follow experimental inoculation of possums with *M. bovis* (Bolliger and Bolliger, 1948; Corner and Presidente, 1980; 1981; O'Hara *et al.*, 1976; Buddle *et al.*, 1994), or oral dosing with tuberculous material(Bolliger and Bolliger, 1948), but experimentally induced infections may not accurately represent natural infections in free-living animals. Deaths were recorded in these studies from 25 to 100 days post-inoculation, and the rapidity of the course of the disease was considered to be dependent on the size of the infective dose. In recent studies, Buddle *et al.* (1994) and Pfeffer *et al.* (1994) have used intra-tracheal inoculation of comparatively low doses of *M. bovis* to produce infection in possums which more closely resembled field disease.

In experimental infection studies, natural infection was observed to develop in un-inoculated cage mates (Bolliger and Bolliger, 1948; O'Hara *et al*, 1976). O'Hara *et al.*, (1976), Corner and Presidente (1981) and Buddle *et al.* (1994) demonstrated natural transmission of infection, apparently by aerosols, to possums caged separately to, but in the same room as experimentally inoculated possums. Transmission from an experimentally infected female to her offspring which she was suckling was first demonstrated by O'Hara *et al.* (1976) and has since been confirmed experimentally by Buddle (pers. comm.). It has also been recognized in multiple animals in the Castlepoint longitudinal study (Pfeiffer and Morris, 1991).

Lesions in experimentally infected animals resembled those seen in tuberculous possums in the wild but the progression of the disease to death was more uniformly rapid. In the Castlepoint longitudinal study of tuberculosis in a naturally infected possum population, the majority of possums died within two months of detection of infection, but a small proportion survived for between six and 22 months following clinical detection of enlarged superficial lymph nodes

culturally positive for *M. bovis*(Pfeiffer, 1994) (Morris, pers. comm.), and must have been infected for some time before the disease became clinically detectable. The rapid progression of disease in experimentally infected possums is probably due to combined effects of a high infective dose, the route of administration and stress induced by housing animals captured from the wild.

Previous survey-style investigations of possum populations have been designed primarily to determine the existence or prevalence of disease in particular possum populations and have not consistently or completely described the total distribution of lesions. In such studies (Julian, 1981; Coleman, 1988; Anon, 1986; Cook, 1975; Lake, 1974; Hickling *et. al.* 1991), gross lesion prevalences varied from 0.53 to 0.81 for lung and 0.23 to 0.54 for superficial lymph nodes, and single site gross lesions varied from 0.31 to 0.57, while in the Hauhungaroa Ranges study the prevalence of lesions in superficial lymph nodes with fistulae was 0.55 (Anon, 1986; Hickling et al., 1991).

However the level of detail provided in these studies is inadequate to formulate robust hypotheses about the pathogenesis of the disease, or about routes of transmission. The following investigation was designed to study the naturally occurring disease in more depth, using standardised procedures developed for the purpose.

MATERIALS AND METHODS

Data was gathered from cross-sectional studies carried out on three separate occasions in mixed pasture, scrub and indigenous forest at a farm (Waio) at Castlepoint in the Wairarapa, on two separate occasions (August 1992 and 1993) on indigenous forest scrub margins at Flagstaff Flat in the Ahaura Valley in Westland (Coleman *et. al.*, 1994), and on another occasion (December 1992) in indigenous forest at the Hochstetter State Forest adjacent to Flagstaff Flat (Coleman *et. al.*, 1993). Where appropriate, findings have also been included from three terminally ill possums obtained from three separate farms in the Wairarapa and from possums obtained during a five year longitudinal study of a naturally infected possum population at Waio (Pfeiffer and Morris, 1991). Since April 1989, the possum population at Waio has been trapped monthly and individuals identified and examined for the presence of infection with *M. bovis* before release. Possums with clinical evidence of tuberculosis had radio-collars fitted and were regularly tracked to their denning locations. Some possums which died from tuberculosis were recovered in a sufficiently fresh condition to allow detailed necropsies to be carried out. These animals were considered part of the terminally ill group. Some possums, commonly immature animals, showed clinical signs of starvation and exposure during prolonged periods of wet and cold weather and died in or near a trap during monthly trapping. Occasionally others died following routine cardiac puncture. Most of these animals were available for necropsy and they are referred to as the misadventure group in this report. In the Wairarapa, farmers and individuals who found possums exhibiting unusual daytime behaviour and signs of illness were encouraged to submit those animals to Ministry of Agriculture and Fisheries (MAF) personnel for detailed examination. Three submitted possums were found to have extensive lesions of generalised tuberculosis and were made available for further detailed examination.

In this and companion papers (Cooke et al.,1995; Jackson et al.,1995), a lesion site is defined as an anatomically distinguishable organ or lymphocentre which contained gross and/or histological evidence of tuberculosis. The term lymphocentre is used as a collective term for a group of lymph nodes at a particular site whereas lymph node is used where there is only a single node. Thus, the axillary lymphocentre includes the single superficial axillary lymph node and the multiple deep axillary lymph nodes (deep axillary lymphocentre). The inguinal lymphocentres describe the multiple inguinal lymph nodes which occur in each side of the inguinal region.

Necropsy and data recording procedures

A protocol (Coleman *et al.*, 1994) was developed at Waio for necropsy procedure, data recording and specimen collection, and was followed in all subsequent field studies with minor modifications to suit specific circumstances (see Appendix 1). Forty standard body sites were sampled for histological examination, (Cooke *et al.* (unpublished). Possums were classified as having no gross lesion (NGL) if no lesions of tuberculosis were visible at necropsy but the animal was later confirmed as tuberculous from histopathology or microbiology.

Female possums were classified as immature if the pouch was not fully developed, and males were classified as immature if testicle width was less than or equal to 13 mm. The right mandible was removed and stored in 10% neutral buffered formalin for later age estimation from the cementum annuli of the second molar, using the method developed by Pekelharing (1970). The clotted blood samples were centrifuged and the serum removed and stored at -80° C for potential future study.

Selection of specimens for bacteriology

With the exceptions of the Flagstaff forest study in December 1992 and the longitudinal study, specimen collection was as follows:

Representative samples of gross lesions (usually a lymph node, but occasionally aspirated content or sterile swabs in transport medium) were stored in a chilled state until they could be frozen at the end of each day. They were kept frozen at -20° C or -80° C until sent to the Central Animal Health Laboratory at Wallaceville for mycobacterial culture. A range of lymph nodes was collected from randomly selected animals with no gross lesions and stored similarly prior to microbiology.

No bacteriological examinations were made for *M. bovis* during the prevalence study in the Hochstetter State Forest adjacent to Flagstaff Flat in December 1992. Diagnosis of tuberculosis in that study was based on gross lesions at necropsy with confirmation by histopathological examination of lesions and multiple tissues.

Bacteriology and histopathology

All bacteriological examinations were carried using the procedures described by Buddle *et al.* (1994). The protocol for selection of specimens for histopathology and their subsequent examination are described by Cooke *et al.* (unpublished).

Waio studies; March 1992, July and September 1993

In these studies, possums were trapped at Waio, Castlepoint, in a valley located about 1 km from the longitudinal study site. Size 1 and 1½ Victor Soft Catch Wildlife Traps lured with a mixture of flour and cinnamon were set at locations which showed signs of movement along "runs", evidence of territorial marking or feeding activities.

In later Waio studies, trapping effort was concentrated around bait stations pre-fed with cinnamon flavoured cereal pellets. Cage traps were also used in the September 1993 study, which was concentrated in and about a gully from which a tuberculous possum was taken during the July 1993 study. In all studies, captured possums were killed humanely, a blood sample collected and the carcases taken to a central place for necropsy.

Statistical Analysis

A range of statistical methods has been used on the data, and each method is stated briefly before results dependent on it are described. Unless otherwise stated, the software used to produce the analyses was Statistica for Windows (Statsoft Inc., 2325 East 13th Street, Tulsa, USA).

RESULTS

Because possums had to be obtained in various ways and under varying circumstances to provide a spectrum of disease pathology, the number of animals included in each part of this paper and following papers has been determined by decisions which allowed all eligible animals to be included in each analysis, but excluded animals for which critical data for a particular analysis was unavailable or uncertain. The number of animals reported therefore varies between analyses.

Point prevalence studies

Four hundred and eighty-six possums were necropsied and examined for presence of tuberculosis. Prevalences and classification of 78 tuberculous possums as animals with and without gross lesions are shown in Table 5.1. Confirmation of tuberculosis and the distribution of lesions were determined by histopathology in 5 of 10 NGL animals. The remaining 5 NGL possums were diagnosed as such by culture of pooled lymph nodes, and hence no lesion distribution was available. Two partly autolysed possums were found: one had gross lesions from which *M. bovis* was cultured but overall lesion distribution could not be determined, the second was confirmed tuberculous by histopathology. Thus 7 animals from the cross-sectional studies were excluded from the analysis of lesion site distribution, leaving 71 animals. However, gross and microscopic lesions of tuberculosis were observed and *M.bovis* isolated in an additional 2 possums which died by misadventure during examination, without clinical signs of disease. These 2 possums are included in the analysis of lesion distribution, making a total of 73 possums.

Table 5.1. Summary of prevalences from field surveys

		Number confirmed tuberculous		
Study	Number necropsied	Gross lesions	NGLs[§]	Total Prevalence*
Flagstaff August 1992	68	35	6	0.6 (0.48-0.72)
Flagstaff December 1992	119	22	2	0.20 (0.13-0.29)
Flagstaff August 1993	54	7	2	0.17 (0.08-0.29)
Waio March 1992	78	0	0	0
Waio July 1993	104	1	0	0.009 (0.002-0.05)
Waio September 1993	63	3	0	0.048 (0.01-0.13)
TOTALS	486	68	10	na

na = not applicable

*Total prevalence ( ) are values of exact binomial 95% confidence intervals

[§]Animals with no gross lesions, subsequently shown to be infected

Table 5.2. Prevalences of gross lesions and gross and microscopic lesions at body sites in 73 tuberculous possums with gross or microscopic lesions of tuberculosis

	Gross lesions		Gross plus microscopic lesions		*Number of
Location	Number	Prevalence	Numbe r	Prevalence	observations
Left superficial axillary Right superficial axillary	30 11	0.411 0.151	43 31	0.597 0.484	72 64
Left deep axillary Right deep axillary	15 9	0.205 0.123	46 34	0.676 0.5	68 68
Left inguinal Right inguinal	14 12	0.192 0.164	37 39	0.521 0.565	71 69
Left tonsil Right tonsil	0 0		16 11	0.219 0.157	73 70
Left mandibular Right mandibular	1 0	0.014	10 10	0.179 0.189	56 53
Left parotid Right parotid	0 1	0.014	5 8	0.083 0.140	60 57
Left deep cervical Right deep cervical	1 3	0.014 0.041	16 15	0.271 0.246	59 61
Left superficial cervical Right superficial cervical	1 0	0.014	9 14	0.150 0.233	60 60
Lung	55	0.753	62	0.849	73
Left anterior mediastinal Right anterior mediastinal	13 12	0.178 0.164	49 49	0.681 0.731	72 67
Mesenteric	19	0.26	43	0.606	71
Gastric	0		17	0.243	70
Hepatic	18	0.247	48	0.686	70
Liver	41	0.562	53	0.726	73
Left kidney Right kidney	17 25	0.232 0.342	25 28	0.352 0.483	71 58
Spleen	15	0.205	36	0.493	73
Bone marrow	0		17	0.277	61
Left adrenal Right adrenal	1 0	0.014	12 8	0.164 0.123	73 65
Left mammary gland Right mammary gland	0 0		2 3	0.077 0.130	26 23
Thymus	0		1	0.015	65

* Number of observations refers to microscopic examinations. All sites were examined for presence of gross lesions and the denominator used for calculation of prevalence of gross lesions was 73. For gross plus microscopic lesions the denominator used was the value in the right hand column, listed under Number of observations.

Distribution of gross and microscopic lesions

Gross lesions of tuberculosis were detected in 68 possums during cross-sectional studies. Supporting evidence of disease came from culture tests for *M. bovis,* histopathology with acid fast organisms (AFOs) in typical lesions, or a combination of both methods. The prevalences of gross lesions and gross plus microscopic lesions at particular organ sites and the number of sites examined are shown in Table 5.2.

The number of gross lesions per possum varied from 1 to 10, with a mean of 4.6. Histopathology of organs increased the total number of lesion sites detected per possum to a mean of 11.6, with a range of one to 28. The correlation between the numbers of gross lesions and total (gross plus microscopic) lesions per individual possum was +0.8.

Table 5.2 also shows the degree of symmetry of lesion distribution between tissues from the right and left sides of the body. The recovery of lymph nodes of the head and anterior neck was reduced and fewer from that region were examined histologically than from more accessible sites, due to their small size when unaffected by gross lesions, combined with the effects of the method of euthanasia.

Liver, hepatic lymph nodes, kidneys, adrenal glands, spleen and bone marrow are considered as a group because lesions in any of these locations indicate haematogenous spread of infection, whereas lesions elsewhere could result from initial infection, lymphatic spread, or haematogenous spread.

The distribution of microscopic lesions was recorded for an additional 5 NGL possums. The addition of data from these 5 possums to data from possums with gross lesions gives a total of 73 possums for which extensive histopathological data is available. These 73 possums comprised 29 females and 44 males.

The distributions of gross and total lesions per possum were tested for normality by the Kolmogorov-Smirnov (K.S.) test conducted in Number Cruncher Statistical System (NCSS) Version 5.03 (Dr. Jerry L. Hintze, Kaysville, Utah, USA) which compares the maximum deviation (K.S. statistic) to a critical value(0.104) with an alpha level of 0.05. Gross plus microscopic lesions were normally distributed (K.S. test value 0.077, <0.104) but gross lesions did not follow a normal distribution (K.S. test value 0.136, > 0.104). The distributions are

illustrated in Figures 5.1 and 5.2. Coefficients of skew for gross and total lesions per possum were 0.21 and 0.26 respectively. In other words, most animals have less than the mean number of lesions, but a small proportion have well above the average number of lesion sites, for both gross and total lesion numbers.

The frequency of occurrence of gross and total lesions in particular groups of lymph nodes and groups of organs are presented in Table 5.3.

Table 5.3. Prevalences of gross lesions and gross plus microscopic lesions at grouped anatomical sites in 73 possums with lesions of tuberculosis

	Gross lesions		Gross plus microscopic lesions	
Anatomical location	Number	Prevalence	Number	Prevalence
Superficial axillary nodes	35	0.479	52	0.712
Deep axillary lymphocentres	24	0.329	57	0.781
Axillary lymphocentres	50	0.685	63	0.863
Inguinal lymphocentres	24	0.329	49	0.671
Head and neck nodes	5	0.068	41	0.562
Lung	55	0.753	62	0.849
Anterior mediastinal	20	0.274	54	0.74
Lung and. mediastinal	56	0.767	62	0.849
Mesenteric and gastric nodes	19	0.26	44	0.603
Liver	41	0.562	53	0.726
Spleen and bone marrow	15	0.205	38	0.521
Kidneys	28	0.384	33	0.452
Liver + hepatic nodes + kidneys + spleen + adrenals +bone marrow	49	0.671	63	0.86
Axillaries + inguinals	55	0.753	69	0.945
Axillaries + inguinals + lung	67	0.918	73	1.0
Axillaries + inguinals + lung + anterior mediastinal	68	0.932	73	1.0

The value of the denominator used in calculations of prevalences of gross lesions and gross plus microscopic lesions was 73.

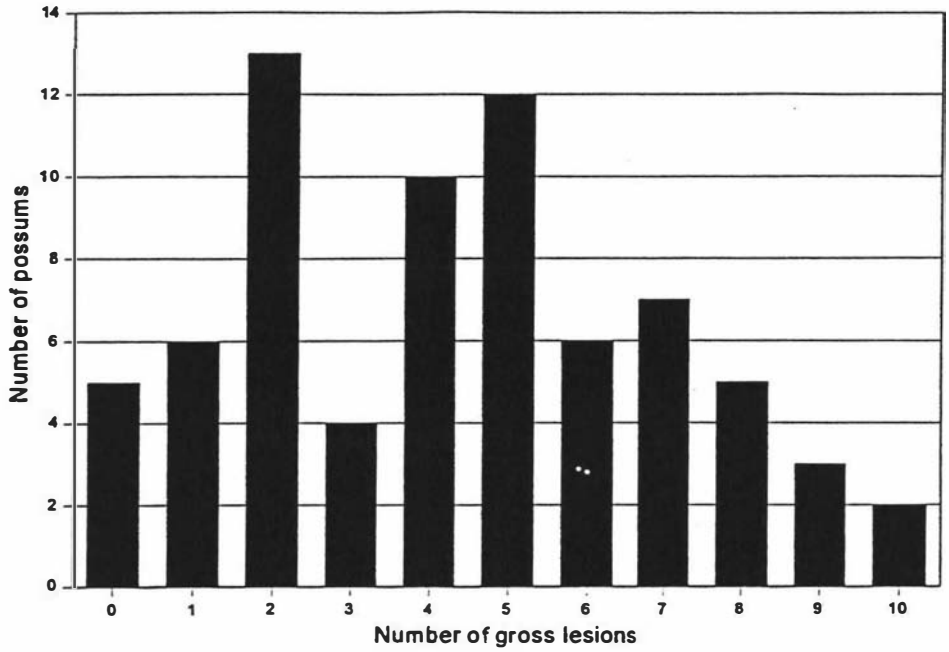


Figure 5.1. Frequency of number of sites containing gross lesions per individual in 73 tuberculous possums.

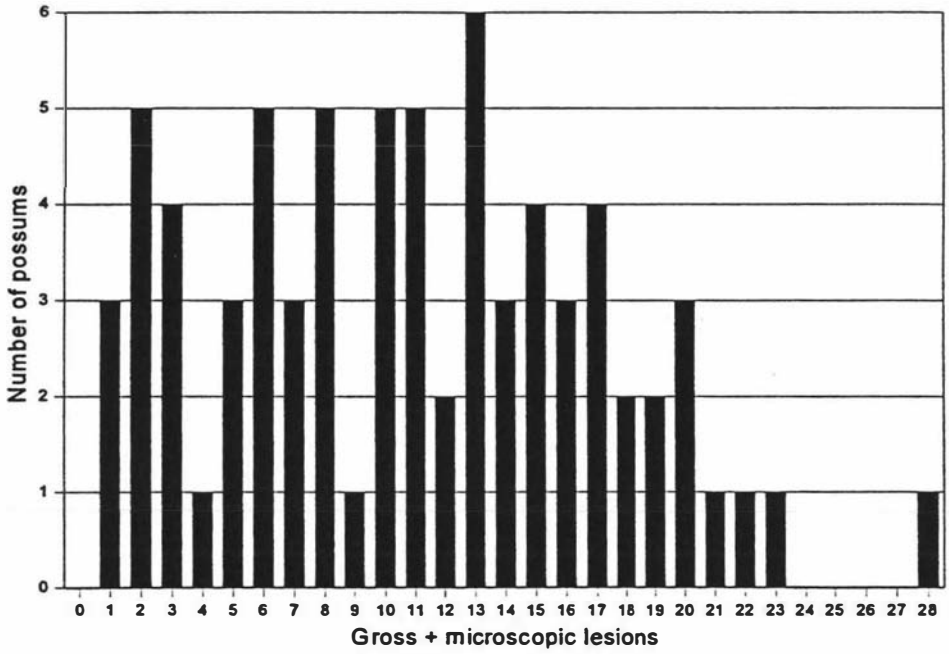


Figure 5.2. Frequency of number of sites containing gross and/or microscopic lesions per individual in 73 tuberculous possums.

Test for symmetry of lesion distribution between both sides of the body

The symmetry of occurrence of lesions between bilaterally located anatomical sites was tested by use of McNemar's χ^2 test. This test is a modification of the $^2\chi$ test and takes correlation between observations on the same individuals into account. Test results are shown in Table 5.4. The left deep axillary lymphocentre shows a disproportionately high number of infections compared with its counterpart, and the left tonsil also shows more infection, although the number of cases is too low for this second conclusion to be drawn firmly.

Table 5.4. McNemar's Chi-squared test values for symmetry of lesion distributions

Anatomical locations	McNemar's χ^2 value differences left and right sides	p-values
Superficial axillary lymph nodes	3.12	0.08
Deep axillary lymphocentres	6.76	0.01
Inguinal lymphocentres	0.05	0.82
Tonsils#	3.27	0.07
Mandibular lymph nodes#	0.1	0.75
Parotid lymphocentres#	2.29	0.13
Deep cervical lymph nodes	0	
Superficial cervical lymph nodes#	0.36	0.54
Kidney#	1.45	0.23
Adrenal#	2.29	0.13
Anterior mediastinal lymphocentres	0	

tables contained cells with expected values less than 5

Association between number of lesioned sites per individual and individual characteristics

The Poisson regression procedure of Statistix (version 4.0 Analytical Software, 4080 Roscrea Drive, Tallahassee FL 32317, USA) was used to test for relationships in tuberculous possums between the number of lesion sites per individual (dependent variable) and the following independent variables: maturity (adult/juvenile), age in years, sex, presence of pouch young, body weight, mesenteric fat weight, presence of lesions in the axillary plus inguinal lymphocentres, presence of lung lesions and capture location effect. Simple Poisson regression was used initially to screen for two-way relationships, with selection for multivariable analysis of those variables which had p-values <0.1 between the response variable and each independent variable except location. Results of the simple regression analyses presented in Table 5.5 show no significant

associations between number of lesion sites and mesenteric fat weight, sex or presence of pouch young.

Four dummy variables were created to enable separation of any effect of differences among the four sources of samples of tuberculous possums used in the analysis (Flagstaff August 1992, Flagstaff December 1992, Flagstaff August 1993 and Waio) from the effect of specific independent variables of interest. Backwards stepwise elimination was used to build the chosen final model. All of the variables listed in Table 5.5 were included. The dichotomous variable, MATURITY, (mature or immature) was dropped from the final model presented in Table 5.6 in favour of YEARS (age in years), despite lower p-values, because YEARS was considered to contribute more definitive information to the model. Table 5.7 presents the results of stepwise analysis of deviance used to assess goodness of fit of the final model. This shows that the presence of lung lesions is the single best predictor of the number of lesion sites per infected possum, with age in years being second in predictive value, although not reaching the 5% level of statistical significance.

Table 5.5. Summary results from initial simple Poisson regression screening analyses for number of lesions per individual

Predictor variable	Intercept value	Slope value	p-value	Deviance	Cases included	Missing cases
Age (Mature-Immature)	2.00	0.481	<0.001	252.98	72	1
Mesenteric fat	2.33	0.008	0.24	216.49	61	10
Sex	2.39	0.003	0.96	283.83	73	0
Years (age in years)	2.30	0.026	0.09	281.00	73	0
P-Y presence	2.42	-0.01	0.89	209.35	53	20
Lung lesions	1.52	1.03	<0.001	185.95	73	0
AX + ING	2.02	0.46	<0.001	257.01	73	0

P-Y = pouch young
AX + ING = axillary + inguinal lymphocentres

Table 5.6. Unweighted Poisson regression of number of lesions per individual for 73 cases

Predictor variables	Coefficient	Standard error	Coeff / SE	p-value
Constant	1.43	0.13	11.30	<0.001
Lung	1.03	0.12	8.64	<0.001
Years (Age in years)	0.03	0.02	1.72	0.09

Deviance 183.05 p-value <0.001 degrees of freedom 70

Table 5.7. Analysis of deviance for goodness of fit in predicting lesion numbers

Model	Deviance	Difference	df	Component	p-value
Intercept (I)	d_1 = 283.83	d_1-d_{ILY} = 100.78	2	LUNG and YEARS	<0.001
I+LUNG	d_{IL} = 185.95	d_{IL}-d_{ILY} = 2.9	1	YEARS	<0.1, >0.05
I+YEARS	d_{IY} = 281	d_{IY}-d_{ILY} = 97.95	1	LUNG	<0.001
I+LUNG+YEARS	d_{ILY} = 183.05				

df = degrees of freedom

Associations between occurrence of lesions of tuberculosis among specific body regions

Associations between the occurrence of lesions at five body regions were examined. The designated regions were the axillary lymphocentres (AXILL), the inguinal lymphocentres (INGS), the head and neck lymph nodes comprising parotid, mandibular, superficial and deep cervical nodes, plus palatine tonsils (HEADNECK), lung and anterior mediastinal lymph nodes (LUNG), and liver, kidney, spleen, bone marrow, adrenal glands and hepatic lymph nodes (HAEM).

Log-linear methodology was used to analyse the cross-classification table formed by the 5 variables. This method allows relationships to be investigated in multi-dimensional contingency tables. Sixty-nine cases were selected from the sample of 73 tuberculous possums, based on adequacy of gross and microscopic lesion occurrence data at the designated sites. The initial model was selected using the automatic selection facility in Statistica which uses a backwards elimination process. The program first fits a model with no relationship between variables, then tests for all two-way and three-way interactions and includes those which improve the fit of the model (the respective χ^2 statistic is significant). All interactions which are not significant are excluded to give a final model with the least number of interactions to fit the observed table.

Variables included in the test were AXILL (1), INGS (2), HEADNECK (3), LUNG (4) and HAEM (5). The best fitting log-linear model was:

[HAEM x INGS] [LUNG x HEADNECK] [HAEM x AXILL] [HAEM x LUNG]

which had a significance level for goodness of fit of 0.99 ($\chi_{22}^2 = 10.00$). Goodness of fit was tested by the χ^2 test and by plotting observed against fitted frequencies. The χ^2 statistic shows a high level of fit for the terms included and a graph of observed versus fitted frequencies showed no major outliers in the table.

The values in the marginal tables calculated by Statistica were analysed using Statcalc (Epi Info Version 5, USD Incorporated, Stone Mountain, Georgia, USA) to examine the nature of the association between the variables for each association included in the model. The calculated Relative Risk statistics are presented in Table 5.8 and show that animals which have lesions in axillary or inguinal lymph nodes or in lung are 1.4 to 1.6 times as likely to have evidence of haematogenous spread as animals which do not. Animals which have head or neck nodes involved are 2.6 times as likely to have lung lesions as those which do not. Other putative infection links investigated did not reach statistical significance.

Table 5.8. Relative risk values for associations between response and design variables in predicting number of lesions per individual

Response variable	Design variable	Relative Risk	Confidence limits	p value
HAEM	AXILL	1.39	0.95 - 2.04	0.04[a]
LUNG	HEADNECK	2.57	1.09 - 6.04	0.006[b]
HAEM	INGS	1.61	0.94 - 2.76	0.06[b]
HAEM	LUNG	1.44	0.99 - 2.1	0.01[a]

[a] Fishers exact 1-tailed p-values
[b] Yates corrected p-values

Terminally ill possums

Eleven possums which either died from advanced tuberculosis or were euthanased *in extremis*, were examined. For 5 of the 11, a wide range of tissues from the list of standard sites was examined histologically. Summary statistics for the frequency of gross and total lesions in individual animals are presented in Table 5.9. Terminally ill possums had a higher mean number

of gross and total lesions than the cross-sectional study possums, which were at varying stages of the disease process.

Because the distribution of lesion numbers is not normal, the Mann-Whitney-Wilcoxon rank sum nonparametric method for comparing equality of medians was used to compare both gross and total lesion numbers between the terminally ill (N=11) and cross-sectional samples of tuberculous possums (N=73). The distributions of frequency of gross and total lesions in the terminally ill groups were significantly different ($p = 0.004$ and $p = 0.03$ respectively) from the distribution in the cross-sectional sample of tuberculous possums. The distributions of the number of gross lesions per individual for both groups are shown in Figure 5.3 and the distributions of total lesions per individual for both groups are shown in Figure 5.4.

Table 5.9. Summary statistics for frequency of gross and gross plus microscopic lesions per individual in terminally ill tuberculous possums

Type of lesion	*Mean	SD	Min	1st Q	Median	3rd Q	Max
Gross (N=11)	7.9(5.2-10.6)	4	2	6	7	12	14
Gross + micro(N=5)	17(12-22)	4	12	12	17	17	23

*Mean ( ) values are 95% confidence intervals

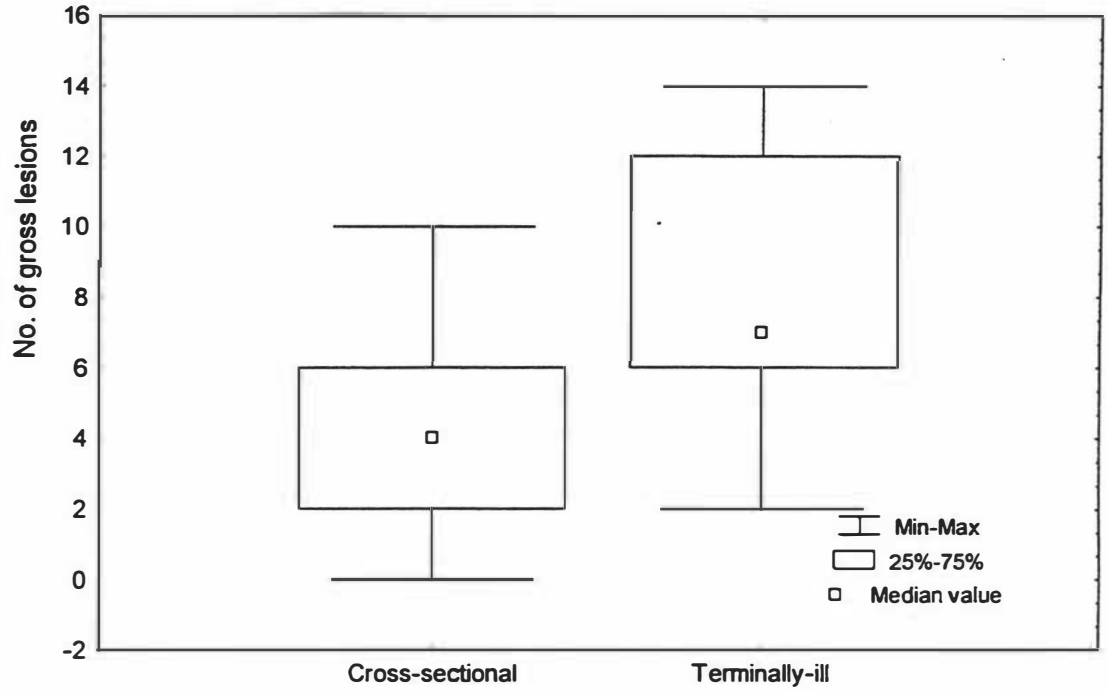


Figure 5.3. Box plot of distributions of gross lesions of cross-sectional and terminally ill groups of possums.

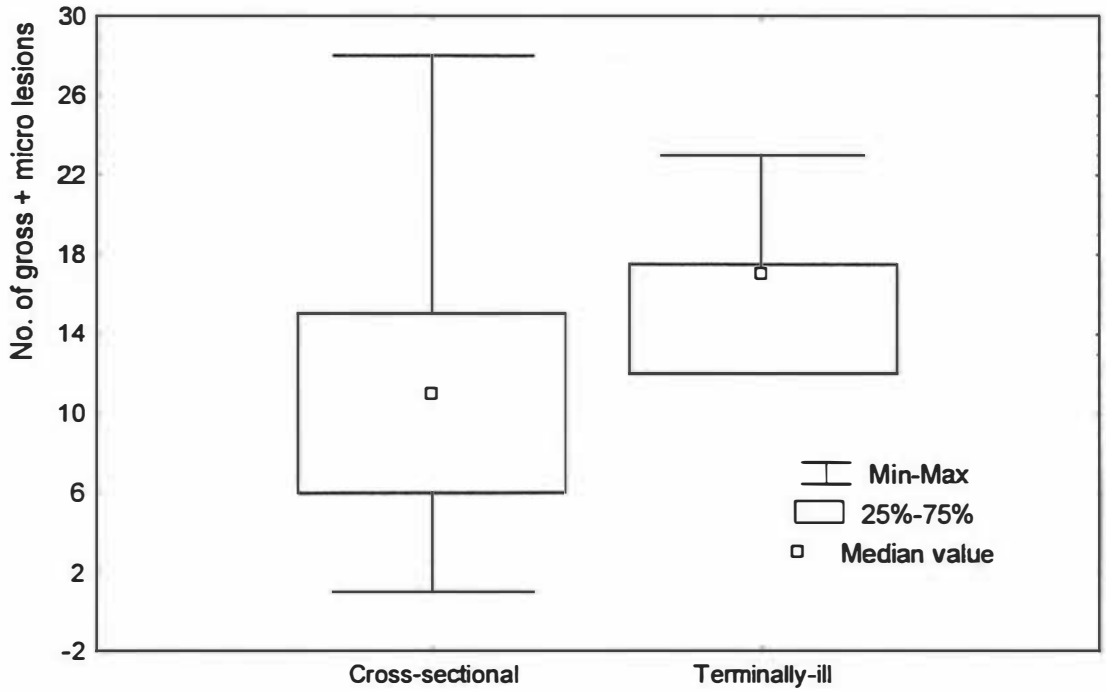


Figure 5.4. Box plot of distributions of gross plus microscopic lesions of cross-sectional and terminally ill groups of possums.

Tuberculous possums with no gross lesions at necropsy

In all studies, randomly selected animals which showed no gross lesions of tuberculosis (necropsy negative) were tested for evidence of tuberculosis using culture or histopathology.

During the Waio 1992 study, tissue samples from organs with evidence of miscellaneous gross pathology were collected from 21 possums for histopathology. There was no microscopic evidence of lesions of tuberculosis and no AFOs were seen in any of the sections.

During the Waio study of July 1993, separate pools of lymph nodes from 13 randomly selected necropsy negative possums were cultured. The pools were subdivided in seven of these animals into a group of superficial nodes, comprising the axillary and inguinal lymphocentres and the superficial cervical lymph node, and a group of deep nodes, comprising the mesenteric, bronchial, hepatic and deep cervical lymph nodes. All culture tests on these and seven of eight randomly selected necropsy negative possums examined similarly during the Waio study of September 1993 were negative.

Mycobacterium bovis was cultured from three of nine separate pools of lymph nodes collected from randomly selected necropsy negative possums during the Flagstaff study of August 1992. One of a further six similarly selected possums showed histopathological evidence of tuberculosis infection. Two of four possums classed as suspicious at necropsy showed histopathological evidence of infection and *M. bovis* was cultured from one of these.

Nine randomly selected necropsy negative possums were examined histopathologically during the Flagstaff study of December 1992. Two showed microscopic evidence of infection with tuberculosis.

During the Flagstaff study of August 1993, tissues for histopathological examination from six necropsy negative possums were selected and pooled lymph node samples from the remaining 41 necropsy negative possums taken for mycobacterial culture. All histopathological examinations were negative for tuberculosis but *M.bovis* was recovered from samples from two of the animals selected for culture tests. In addition, *Mycobacterium avium* was cultured from a pooled sample of deep nodes from one possum in this study.

The results of the culture and histopathological tests carried out on necropsy negative animals are presented in Table 5.10.

Table 5.10. Results of histopathology and culture tests for tuberculosis carried out on necropsy negative (NN) possums

Study	Total Prevalence	Number of NNs tested			Number of NNs untested
		Positive	Negative	Prevalence ( )	
Flagstaff Aug.1992	0.6	6	13	0.316 (0.126-0.566)	13
Flagstaff Dec 1992	0.2	2	7	0.222 (0.028-0.60)	89
Flagstaff Aug 1993	0.17	2	45	0.043 (0.005-0.145)	0
Waio March 1992*	0	0	23	0	55
Waio July 1993	0.009	0	13	0	90
Waio Sept 1993	0.048	0	8	0	52
Total		10	109	0.084 (0.041-0.149)	299

Prevalence ( ) exact binomial 95% confidence intervals
* tissues selected were confined to any organ showing evidence of abnormality

Comparisons between tuberculous and non-tuberculous possums

Logistic regression was used to test for associations between the infection status of tuberculosis (dependent variable TB) and individual animal characteristics in the samples of possums from the three Flagstaff studies. The individual animal measurements selected for examination as independent variables were sex (SEX), body weight measured in kilograms (WEIGHT), age in years (AGE) and mesenteric fat weight measured in grams (MES).

Calculations were made using Statistix Version 4.0. Unweighted logistic regression was used initially to screen for two-way relationships with associated p-values <0.1 between the response variable and each of the independent variables. Results of these initial logistic regression analyses are shown in Table 5.11. This shows that of the variables considered, sex was the only one which had significant predictive value for infection status. The proportion of males infected was 37%, and the proportion of females was 20.8%. Relative risk of infection for males was therefore 1.78 (Taylor Series 95% confidence limits of 1.18 to 2.70).

Table 5.11. Summary results from initial logistic regression screening analyses for presence of tuberculosis

Predictor variable	Coefficient	p-value	Deviance	Odds ratio	Cases included	Missing cases
MES	-0.04	0.11	268.63	0.96(0.91-1.01)	232	7
SEX	0.81	0.006	275.21	2.24(1.26-3.99)	238	1
WEIGHT	-0.04	0.83	279.68	0.96(0.65-1.41)	236	3
AGE	0.07	0.23	280.85	1.08(0.95-1.22)	237	2

Comparison of distribution of lesions in lymph nodes and lymphocentres by sex

The distribution of lesions in all lymph nodes and all lymphocentres was compared between tuberculous males and females using the χ^2 test. No significant differences were found.

Comparisons between distributions of gross lesions in possums in this series of studies and previous studies

In the studies described here where detailed examinations of individual possums were made, six of 68 possums with gross lesions had single site gross lesions and 55 of 68 had gross lesions detected in lung. Comparisons with other studies which have reported frequency of single site lesions, lung lesions and superficial node involvements are presented in Table 5.12. The odds ratios listed for single site lesions indicate the chances of finding a possum with a single site lesion in our study, compared with the results reported in other less detailed studies, which for the purposes of comparison are given the value 1.0. A similar analysis is reported for the presence of gross lung lesions. In our studies, significantly fewer single site and more lung lesions were recorded.

Table 5.12. Comparison of frequency of gross lesion occurrence in studies reported here with values from previously reported studies

Study	Odds ratio[§]	Confidence limits	p-value
Single sites			
Coleman (1988)	0.08	0.02 - 0.21	<0.001[a]
Lake (1974)	0.22	0.06 - 0.84	0.02[b]
Hickling *et. al.* (1991)	0.07	0.02 - 0.20	<0.001[a]
Lungs			
Coleman 1988)	3.40	1.55 - 7.56	0.001[a]
Lake (1974)	1.01	0.25 - 3.5	1[b]
Hickling et. al. (1991)	3.81	1.69 - 8.7	<0.001[a]
Cook (1975)	2.28	1.09 - 4.83	0.026[a]

[a] denotes Yates corrected p - value [b] denotes Fishers exact 2 - tailed p - values

[§] Odds ratio for the present study compared with cited earlier papers, set to 1.0

DISCUSSION

As far as is known, this study is the first to apply quantitative epidemiological methods to a disease, using a large sample of animals subjected to intensive pathological evaluation. The aim has been to reconstruct the process of pathogenesis in the absence of any *in vivo* diagnostic test, and to assess the impact of various risk factors on the process. It provides a number of insights not available through investigational methods used previously.

For these investigations, diagnosis of infection with *M. bovis* was based either on culture of *M. bovis* from samples or on histopathology demonstrating the presence of typical lesions and AFOs, or both. The specificity of culture tests for *M. bovis* is very high provided cross-contamination of samples does not occur, and in these studies, considerable effort went into preventing its occurrence. The sensitivity of culture tests is expected to be very high for aspirates of lesions and for intact gross lesions, but may be reduced when organisms occur sparsely in early lesions, or when tissues are pooled. Freezing causes small losses of viable organisms in tissues, but major reductions can accompany the decontamination process undertaken on samples prior to culture. Care was taken during necropsies to reduce contamination to a minimum in order to allow bacteriology to proceed with minimal decontamination.

The sensitivity of histopathology for diagnosis was maximised by examination of a wide range of tissues from each animal including all visible lymph nodes from lymphocentres where multiple nodes occur. Possums were only classified as histopathologically positive if multiple microscopic granulomatous lesions containing macrophages and AFOs were present. Although AFOs in sections cannot be positively identified as *M. bovis*, infections with other mycobacteria which may be confused with this organism are rare in possums. *Mycobacterium avium* was recovered from one sample (pooled deep lymph nodes) during our studies and previously from one of 121 pooled lymph node samples taken from 126 possums in 1986 (P. Livingstone, pers. comm.). We are confident that the specificity of the histopathology in our studies was very high.

The sensitivity of histopathology for single tissues is limited by a logistical restraint of confining examinations to several 4 μm thick sections of tissue. The reduction in sensitivity was likely to be higher for large organs such as lung or liver than for small lymph nodes under circumstances where gross lesions were absent and microscopic lesions were scarce. Overall, we consider the estimates of prevalence and presence of lesions diagnosed by bacteriology or histology to be mildly conservative.

The possums described here were derived from populations sampled over 18 months. Because of the approach adopted, the sample is believed to include animals at all stages of the disease. Our findings present the most accurate description of the nature of naturally occurring disease to date and allow some inferences to be made about the pathogenesis of the disease in possums.

Multiple gross lesions occurred commonly in tuberculous possums. Only six of 68 possums with gross lesions had those limited to single body sites, and the mean value for the number of grossly affected organ sites per individual was 4.6. This finding contrasts with studies by earlier investigators (see Table 5.12), who reported greater proportions of possums with single site lesions. We also found higher total frequencies of gross lesions than others have done. These differences are attributed to our use of a more detailed necropsy procedure. Furthermore, our use of histopathology revealed that gross lesion frequency distributions considerably under-estimate the true lesion distributions as measured by the frequency of gross plus microscopic lesions. The mean value for total lesions per individual was 11.6. Of the six animals with single site gross lesions, in no case was this the only site once histopathological and cultural results were taken into account. No associations could be shown between the number of lesions per individual and sex, maturity, presence of pouch young, indices of body condition or presence of lesions in superficial lymphocentres. However, possums with lung lesions had more lesion sites than those without lung lesions and there was a suggestion of a weak age effect, with older animals having slightly larger numbers of lesion sites.

No associations were found between disease status and body weight or weight of mesenteric fat depots. Infection was also spread evenly across all ages, but cross-sectional studies such as this one do not provide a valid basis for drawing inferences about the incidence of infection in different age groups - which would require a longitudinal study method. The lack of association between disease status and either body weight or the weight of mesenteric fat depots is in agreement with Coleman *et al.* (1994) but contrasts with earlier analyses by the same author (Coleman 1988). In the 1994 study, Coleman *et al.* (1994) used different analytical techniques from those used here to examine a sub-sample of possums tested by us. The agreement between the analyses of the sub-group and the whole sample increases confidence in both conclusions. The discrepancy between these findings and the earlier ones may be because possible confounding effects of sex and age were not controlled for in the 1988 analysis(Coleman, 1988).

All experimental infection studies have reported weight loss and a decline in body condition with progression of the disease. Pfeffer *et al* (1994) reported onset of weight loss in captive possums

14 days after infection with a low dose (125 colony forming units) of *M. bovis* administered intra-tracheally. This contrasts with findings from the longitudinal study at Castlepoint where sequential observations have indicated that tuberculous possums commonly but not invariably lose weight in the one to two months prior to death. However, up to that point in the disease process, there has been no measurable effect on body condition and many diseased animals continue to grow and thrive. At Castlepoint, diagnosis of disease is based on the presence of palpable increases in size of superficial lymph nodes. It is likely that such animals are already infected at multiple body sites, although accurate determination of time of initial infection has not been possible. Although the period from detection of disease until death in such animals is usually less than three months (Pfeiffer, 1994), animals have survived for over a year following initial detection of disease in lymph nodes. Moreover, such animals must have been infected for a significant period of time before lesions were first detected by palpation. Although lack of an accurate diagnostic test has made it impossible to assess the length of this subclinical period, when animals on the Castlepoint study site were killed and examined at the end of the study, a number were culture positive without gross lesions, under circumstances where the evidence suggested that initiation of infection had been probably as much as 12 to 22 months earlier (I. Lugton, pers. comm.).

Lesions in liver, spleen, kidneys, adrenal glands and bone marrow are considered indicative of generalised infection and almost certainly have resulted from haematogenous spread of AFOs either as free bacilli or intracellularly. We found lesions in one or more of these sites in 86% of cross-sectional study possums indicating that haematogenous spread of disease is common and occurs relatively early in the disease process. Log-linear analysis showed that animals with infection in lung and axillary or inguinal lymphocentres are more likely to have evidence of haematogenous spread than animals which do not have these lesions. Lesions were found in the lungs in 84% of tuberculous possums.

The typical form which the disease assumes in affected animals involves establishment of infection at one or more initial sites, followed by rapid extension to other body sites, often by the haematogenous route. It would seem that spread to multiple sites commonly occurs before lesions become visible at any body site, since significant numbers of necropsy negative animals had multiple lesion sites, while only two animals in the entire study had single site lesions. The highest prevalence of infection was found in lung, and in axillary and inguinal lymphocentres. Although this might suggest a primary role for these sites in the disease process, the evidence allows only the formulation of hypotheses on such issues, not their testing. Apart from a

suggestion of a weak age effect on the number of affected organ sites, the pathology and pathogenesis of the disease appeared unaffected by sex and maturity. The nature and appearance of microscopic lesions indicates a partly effective host response which is unable to wall off established lesions and allows formation of satellite lesions about and close to established central lesions (Cooke *et. al.*, submitted). Despite this apparently limited host response which allows dispersal of organisms and consequent development of lesions in multiple organ sites, normal growth and behaviour of the possum are unaffected until the terminal stages. Lesions of tuberculosis are a direct result of the immune response by the host to antigens presented by *M. bovis* organisms. Mycobacteria are not toxigenic and dysfunction probably only becomes apparent when there is interference with function from expanding and obliterating lesions, or the toxic products of tissue breakdown from extensive lesions interfere with metabolic processes of the body, akin to advanced neoplastic disease. In field cases of tuberculosis in the possum, the animal appears to withstand those effects for a lengthy period even after there are extensive gross lesions, then a threshold is reached where the metabolic homeostasis of the animal is overwhelmed, a catabolic state sets in and there is rapid decline, cachexia and death. A combination of high prevalence of lung lesions and generalised disease in a highly susceptible host, whose behaviour is unaffected until a terminal stage, is ideal for ensuring high levels of contagion.

Although prevalence of disease was higher in males than females in the Flagstaff studies (Relative Risk = 1.78), this apparent sex difference should be interpreted carefully. Approximately equal proportions of males and females were captured, but the trapped population may not have been an accurate representation of the targeted population. Females are known to be more difficult to trap than males (Brockie *et. al.*, 1981; Coleman and Green, 1984) and the differences could be partly due to unequal trappabilities between the sexes, and between infected and uninfected animals of each sex, despite the use of intensive trapping methods. During the first 52 month period in the longitudinal study at Castlepoint, 390 males and 244 females were individually identified and of those possums, similar proportions (36 males and 25 females) were diagnosed as tuberculous. Thus caution is required in interpreting prevalence data from a cross-sectional study where it may be confounded by differences in trappability.

The tuberculous animals examined in this series represent all detectable stages of the disease from subclinically infected animals with microscopic lesions to a stage where there are multiple lesions at a variety of body sites, some of which emerge as grossly detectable, while others remain purely microscopic. Initial lymphatic spread appears to expand into haematogenous

spread unusually early in the course of the disease, compared with other species. From the Castlepoint longitudinal study, progression of the disease seems initially to be slow to imperceptible, but as the number of grossly visible lesions grows, the disease switches at some point from a relatively benign form to a rapidly progressive form and the animals become terminal. The terminally ill group are characterised by widespread proliferative lesions which have gradually replaced normal tissue, particularly lung, accompanied by debility from associated catabolic effects and interference with normal function. This group is potentially highly infectious, but such animals are debilitated and the abnormal behaviour which they can show may change the exposure of other possums and especially domestic livestock to these animals, compared with the situation before they reached the terminal debilitated state.

The cellular response to infection in possums may resemble that found in other species, with great variation between individuals occurring at the stage of dissemination from the primary site. A primary lesion or primary complex can be expected to develop following initial infection. Drawing on evidence from man and other species as well as the possum, and despite the lack in the possum of classical circumscribed granulomas resembling those commonly seen in eutherians such as cattle, the primary lesions within that complex may apparently remain static for some time during a period of latency or incubation, or may increase progressively in size. There is no evidence to date to suggest that lesions of tuberculosis in possums resolve naturally. Generalisation of the disease may occur concurrently with or as a sequel to development of the primary lesion complex. The disease then progresses through a stage of obvious clinically detectable disease to a terminally ill stage which lasts for about two weeks to two months prior to death. The paucity of available information about disease development in possums under natural conditions allows very limited speculation about rates of disease development other than that the time taken for each stage appears to be variable. There is direct and circumstantial evidence from the Castlepoint longitudinal study that the disease can be chronic in nature and very variable in duration of both the interval from infection to identifiable disease, and from that date to death. External factors may well be influential in precipitating the transition from one disease stage to the next.

The high prevalence of lung lesions is probably of central importance to the pathogenesis of the disease. Although palatine tonsils were affected in 26% of tuberculous possums, they were always associated with lung lesions and generalised infection, and almost always with mesenteric lesions and discharging sinuses. It appears most likely that infection in tonsils results from generalisation from other sites rather than as an early sequel to contamination of the oropharynx.

In the possum, deep cervical lymph nodes receive afferent vessels from the oropharynx and tonsils (R. Jackson, pers.obs.), and although all 25 animals with lesions at those lymph nodes had accompanying lung lesions and generalised infection, only 12 of the 25 had accompanying lesions in tonsils. Although lesions in lymph nodes which drain the nasal cavity, oropharynx and digestive system may result from primary infection at those sites, they appear to be either a part of generalised disease or subsidiary to lesions in lung. Evidence for cattle and deer suggest that primary infection of the oropharynx (and hence the retropharyngeal lymph nodes) may be much more common than in the possum, reflecting the aerodynamics of organisms entering the oropharynx of the different species.

Infection of gastric lymphocentres also occurred in association with mesenteric, lung and generalised infections but infection in the gastric lymphocentre was less frequent than in the mesenteric lymphocentre. Mesenteric lymph nodes were commonly affected but they were most frequently associated with lung and generalised infections. These findings are in agreement with the generally held view that organisms deposited in the pharynx by reflux from open lung lesions may lead to local infections at either that site or further down the gastrointestinal tract following swallowing.

The distribution of gross lesions recorded in these studies is relevant to necropsy procedures in field studies designed to determine prevalence of tuberculosis. In our sample of animals, gross examination of both axillary and inguinal lymphocentres would have detected 81% (95% confidence intervals [c.i.] = 70 - 89%) of grossly affected and 75% (64 - 85% c.i.) of all diseased animals. Examination of these lymphocentres together with lung and anterior mediastinal lymph nodes would have detected all (95 - 100% c.i.) grossly affected and 93% (85 - 98% c.i.) of all diseased animals. Considerable savings in time and effort could be made in future if inspections were restricted to the superficial lymphocentres and thoracic contents, and these sites were examined in more detail.

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CHAPTER 6

Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichosurus vulpecula*): III Routes of infection and excretion[1]

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[1] Accepted as Jackson R, Cooke MM, Coleman JD, Morris RS, de Lisle GW, Yates GF. for publication in the New Zealand Veterinary Journal. Several additional tables and figures have been included in this version for completeness of reporting and improved clarity.

ABSTRACT

Mycobacterium bovis was cultured from nine (36%) of 25 tracheal washings but not from any of 38 urine and 38 faecal samples from tuberculous possums cross-sectionally sampled from the wild. One (33%) of three tracheal washings, one of three urine samples and one of three faecal samples from terminally ill possums were culture positive. The respiratory route is implicated as the major route of excretion of *Mycobacterium bovis* from naturally infected possums in horizontal transmission. Tuberculosis was observed in two young possums and was evidence of probable pseudovertical transmission via the respiratory route or ingestion of milk. Discharging fistulae were present in 22 (31%) of 71 cross-sectionally sampled tuberculous possums and were associated with relatively advanced disease. Although the frequent involvement of superficial lymphocentres in early stage disease could not be explained satisfactorily, the respiratory route was implicated as the main route of infection from indirect evidence.

INTRODUCTION

The limited but compelling evidence from the studies of O'Hara *et al.* (1976), Corner and Presidente (1981) and Buddle *et al.* (1994) supports an hypothesis that the respiratory route is the principal route of transmission of tuberculosis in possums which have acquired natural infections while housed under experimental conditions. The two principal routes of infection for tuberculosis in animals and man are respiratory and alimentary, but the relative importance of each route varies between and within species under the influence of factors such as age, nature of diet and behaviour. Although there is no definite proof, there is now general acceptance, based on multiple sources of evidence, that the principal route of infection for cattle is respiratory (Pritchard, 1988).

Although routes of excretion may be determined from relatively simple studies, few studies have provided any data on the subject and none have adequately examined excretion routes in possums. In the wild state, the obvious potential routes of excretion of infective material from possums are via aerosols generated from the respiratory tract, exudate from discharging sinuses, saliva, milk, urine and faeces. In one study (P. G. Livingstone, pers. comm.), organisms were recovered post mortem from the pharynx (19 isolates from 51 pharyngeal swabs from tuberculous possums), faeces (six isolates from 51 specimens) and urine (one isolate from 14 specimens). Although Livingstone suggested that some cross-contamination of samples may have occurred and caused overestimates of positive tests, the study provided a useful guide to the relative importance and frequency of each route of excretion. Direct transmission from mother to offspring has been recorded both under experimental (O'Hara *et al.*, 1976; personal observations by R. Jackson and B. M. Buddle) and natural conditions (Pfeiffer and Morris, 1991).

In some species, including man and cattle, the locations of primary lesions formed at the establishment stage of tuberculosis are considered to be important indicators of possible routes of infection, while the distribution and character of lesions in animals at more advanced stages of the disease are useful indicators of possible routes of excretion of organisms. Although cross-sectional pathogenesis studies cannot provide unequivocal proof of the ways in which disease is initiated and transmitted, they are valuable for producing new hypotheses and for providing information to support or refute theories generated from other avenues of investigation.

In the study reported in this paper, tracheal washings, urine, and faeces were collected from infected possums and examined for the presence of *Mycobacterium bovis*, and hypotheses were developed based on culture tests and pathological evidence from the distribution of lesions reported in companion papers (Jackson *et al.*, submitted; Cooke *et al.*, submitted) about likely routes of infection and excretion.

MATERIAL AND METHODS

The methods of collection of possums and necropsy procedures have been described earlier (Jackson *et al.*, submitted; Cooke *et al.*, submitted). Data was gathered from cross-sectional studies carried out on two separate occasions in mixed pasture scrub and indigenous forest at a farm (Waio) at Castlepoint in the Wairarapa, on two separate occasions (August 1992 and 1993) on indigenous forest scrub margins at Flagstaff Flat in the Ahaura Valley in Westland (Coleman *et al.*, 1994), and on another occasion (December 1992) in indigenous forest at the Hochstetter State Forest adjacent to Flagstaff Flat (Coleman *et al.*, 1993). Where appropriate, findings have also been included from possums obtained during a 5-year longitudinal study of a naturally infected possum population conducted at Waio (Pfeiffer and Morris, 1991), and from three terminally ill possums obtained from three separate farms in the Wairarapa. During postmortem examinations, urine, faeces and tracheal washings were collected from random samples of possums from which tissues were taken for bacteriological and/or histopathological examinations. Urine was collected directly through the bladder wall into vacutainers via sterile needles. Tracheal washings were collected after instilling 3 – 5 ml of sterile normal saline solution via a tomcat catheter through an incision in the trachea and then distributing the saline throughout the air spaces by gently rolling the lungs back and forth prior to retrieving the remaining fluid. All specimens for bacteriology were stored in a frozen state until processed by the Central Animal Health Laboratory at Wallaceville.

All statistical analyses were conducted using Statistica for Windows (Statsoft Inc., 2325 East 13th Street, Tulsa, USA).

Bacteriology

About 1 g of faeces was vigorously vortexed in 40 ml of sterile distilled water and then allowed to stand for 20 minutes to allow the heavy particles to settle. A 5 ml sample of the top layer of

the suspension was added to 35 ml of 1% cetylpyridinium chloride (CPC) and allowed to stand for 30 minutes at room temperature. The sample was centrifuged for 25 minutes at 3500 *g* and the sediment resuspended in about 1 ml of sterile distilled water. The resuspended sediment was then inoculated on two slopes of pyruvate supplemented 7H11 medium, one slope of glycerol supplemented 7H11 medium, one slope of pyruvate supplemented Lowenstein Jensen medium and one slope of glycerol supplemented Lowenstein Jensen medium. Urine was decontaminated by adding 5 ml of it to an equal volume of 1.5% CPC, and then allowed to stand for 20 minutes. The centrifugation conditions and culture media were the same as that used for culturing the faeces. Tracheal washings were cultured using an identical procedure to that for urine except the concentration of CPC was 0.75%. The conditions for culture and identification procedures were the same as those described previously (Buddle *et al*., 1994).

RESULTS

Recovery of *M. bovis* from tracheal washings, urine, faeces and pouch young of tuberculous possums

The results of culture for *M. bovis* from tracheal washings, urine and faeces collected from tuberculous possums are presented in Table 6.1. *Mycobacterium bovis* was cultured from nine (36%) of 25 tracheal washings, but not from any of 38 urine and 38 faecal samples from tuberculous possums cross-sectionally sampled from the wild. One (33%) of 3 tracheal washings, one of three urine and one of three faecal samples taken from three terminally ill possums were culture positive. The presence of *M. bovis* in tracheal washings was supported by multiple histological observations of acid fast organisms (AFOs), both free and within macrophages in airways. Although the overall prevalence of tuberculous lesions in kidneys was 0.45 (Jackson *et al.*, submitted), excretion in urine appeared to be uncommon.

Samples of lung and liver were taken from two pouch young from tuberculous females captured during the cross-sectional studies and submitted for *M. bovis* culture. Both were negative but *M. bovis* was recovered from a sample of lung and liver taken from an about 70-day-old pouch young from a terminally ill female from the longitudinal study. Four (12%) of cross-sectionally sampled tuberculous females had lesions in their mammary glands.

Table 6.1. Results of culture tests for *M. bovis* from tracheal washings, urine and faeces of tuberculous possums

	Tracheal wash		Urine		Faeces	
Study	Positive	Negative	Positive	Negative	Positive	Negative
Flagstaff Aug 1992	8	9	0	28	0	28
Flagstaff Aug 1993	1	3	0	6	0	6
Waio July 1993	0	1	0	1	0	1
Waio Sept 1993	0	3	0	3	0	3
Total (cross-sectional)	9	16	0	38	0	38
Terminally ill	1	2	1	2	1	2
Total (all samples)	10	18	1	40	1	40

Occurrence of fistulae draining tuberculous lymph nodes to the exterior

Fistulae were present in 22 of 71 tuberculous possums. Summary statistics of the number of total lesion sites for possums with and without fistulae are presented in Table 6.2. The data are not normally distributed and the Mann-Whitney-Wilcoxon rank sum test was used to compare the frequency of occurrence of lesion sites in both groups. Most lesions were recorded in possums with fistulae ($Z = -4.863$, $p < 0.001$).

Table 6.2. Summary statistics for frequency of gross plus microscopic lesions per individual in 71 possums with and without discharging fistulae

Presence of fistulae	*Mean	SD	Min	1st Q	Median	3rd Q	Max
Present (N = 22)	16.1 (14.0 - 18.3)	4.81	8	12	15.5	19	28
Absent (N = 49)	8.3 (6.8 - 9.8)	5.22	1	3	8	11.5	20

*Mean ( ) are values of 95% confidence intervals

The distributions of the total lesions sites in the two groups are illustrated in Figure 6.1.

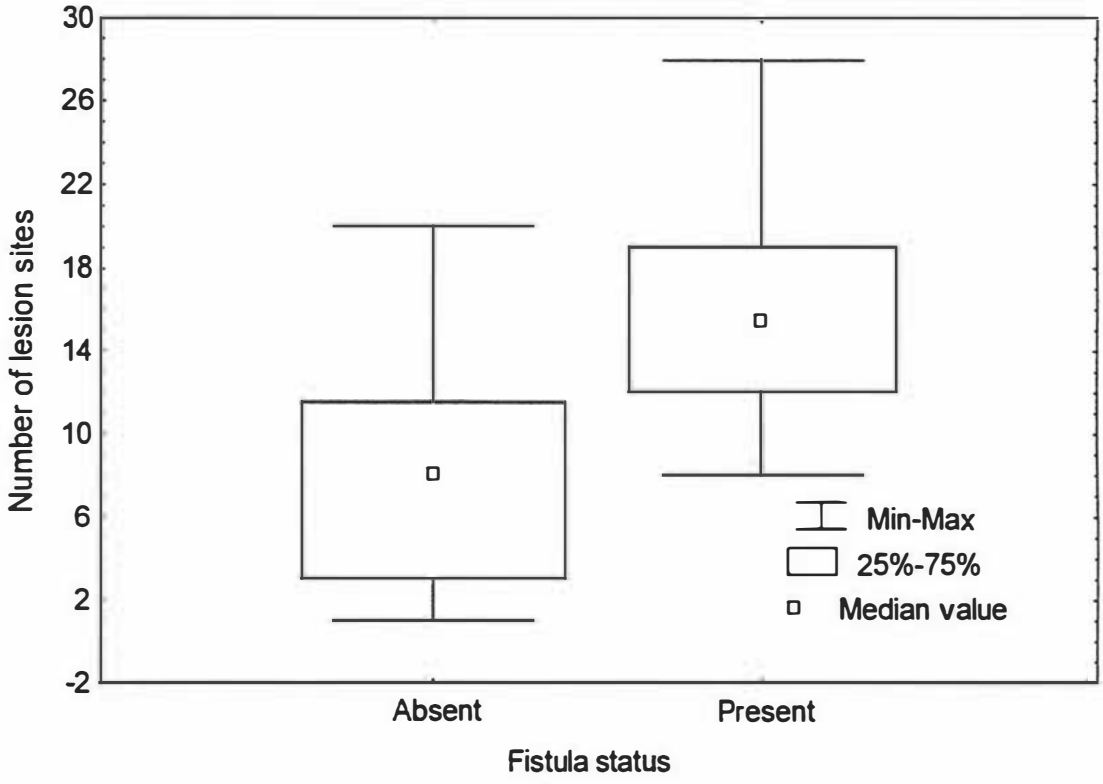

Figure 6.1. Box plot of number of gross plus microscopic lesion sites in tuberculous possums with and without discharging fistulae

The group affected by fistulae was further examined by comparing the number of lung lobes with lesions per individual in affected and non-affected groups. The Mann-Whitney-Wilcoxon rank sum test demonstrated that possums with fistulae had significantly more lung lobes with lesions than animals without fistulae ($Z = -4.587$, $p < 0.001$). Summary statistics for the two groups are set out in Table 6.3 and illustrated in Figure 6.2. Only three possums with discharging fistulae did not have all six lung lobes affected and gross lesions were detected in those animals in one, two and five lobes. All of the group with discharging fistulae featured lesion distributions consistent with generalised infection and it appears likely that, as a group, they represented a relatively advanced stage of disease. All females with lesions in their mammary glands had discharging fistulae.

Table 6.3. Summary statistics for number of lung lobes containing lesions in individual possums with and without discharging fistulae

Presence of fistulae	*Mean	SD	Min	1st Q	Median	3rd Q	Max
Present (N = 22)	5.6 (5.1 - 6.1)	1.18	2	6	6	6	6
Absent (N = 49)	3.0 (2.4 - 3.7)	2.32	0	1	3	5	6

*Mean ( ) are values of 95% confidence intervals

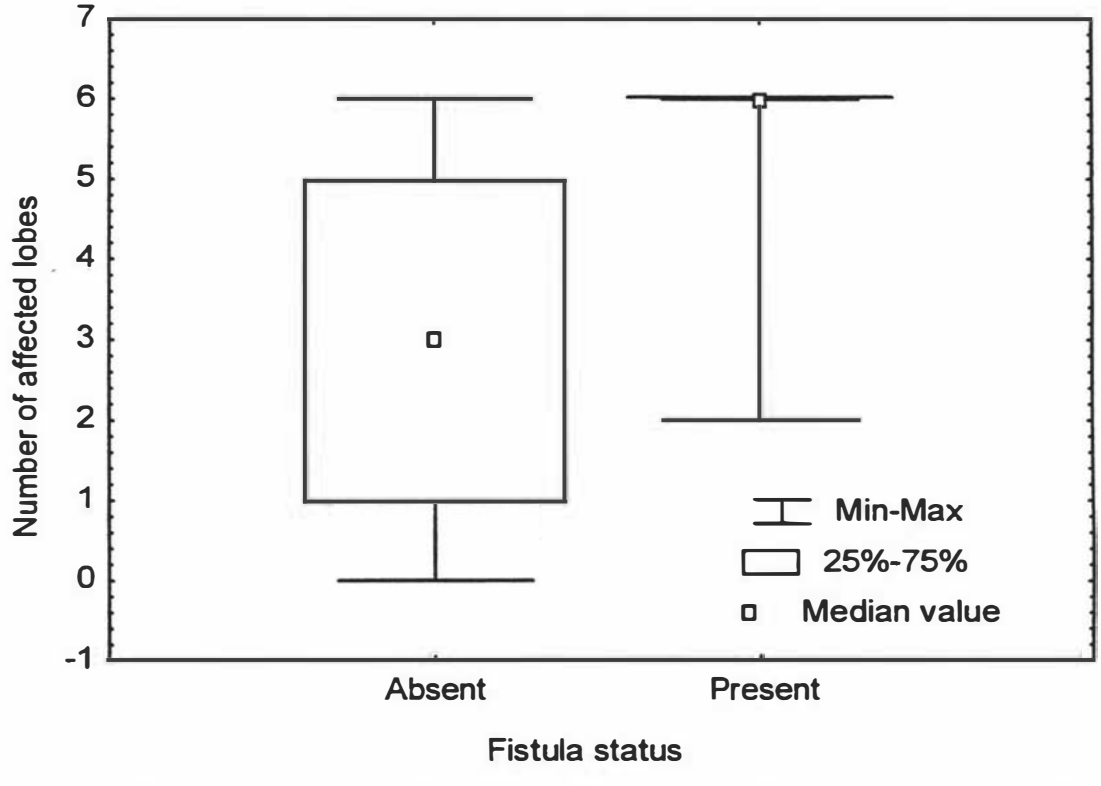

Figure 6.2. Box plot of number of lung lobes with lesions in tuberculous possums with and without discharging fistulae

Disease characteristics in tuberculous possums with lesion distributions consistent with early stage disease

Possums with four or less organ sites affected were given special attention because of the potential value of the distribution of their lesions for producing hypotheses about likely routes of infection. The distribution of lesions of 11 such possums set out in Table 6.4 show a predominance of axillary and inguinal lymphocentre lesions.

Two possums, H3126 and H3132, had not reached the age of independence and were therefore still in intimate contact with their mothers. They would have had virtually no opportunity for contact with other possums outside of the mother-offspring association and it is highly likely that they were infected from their mothers. Only one possum, B6104, showed evidence of infection confined to mesenteric lymph nodes.

In all but one (D5246) of the remaining possums listed in Table 6.4, lesions were confined to superficial lymphocentres and, in this animal, the presence of liver lesions suggests that infection may have been at an early stage of generalisation.

Table 6.4. Distributions of gross plus microscopic lesion sites in possums in which four or fewer lesion sites were detected

			Lesion site [b]									
Identity	Sex[a]	Years	LSA	RSA	LDA	RDA	LI	RI	Ant M	Lung	Mes	Liver
G1100	F	4	+		+							
G1097	F	8					+					
B6113	F	5					+	+				
200S	M	1.5			+		+					
D5241	M	1.3	+	+	+							
B6257	M	7	+	+		+						
B6114	M	1.3	+		+			+				
D5246	F	1			+			+		+		+
H3126	F	0.75			+				+	+		
H3132	M	0.5							+	+		
B6104	F	8									+	

[b]LSA = left superficial axillary lymph node; LI = left inguinal lymphocentre;RSA = right superficial axillary lymph node; RI = right inguinal lymphocentre; LDA = left deep axillary lymphocentre; RDA = right deep axillary lymphocentre; Mes = mesenteric lymphocentre;Ant M =anterior mediastinal lymph node;

[a]M = male;F = female.

The predominance of infection in superficial lymph nodes and the absence of any evidence of primary site infection in cutaneous or subcutaneous tissues which drain to these lymphocentres was a puzzling finding in the remainder of the possums listed in Table 6.4. In possums, all subcutaneous tissues eventually drain either through the superficial cervical or the deep axillary lymphocentres which then drain into the venous system. In an attempt to determine whether infection, undetectable histologically but detectable by culture methods, also occurred in visceral organs, separate collections of pooled lymph node samples were taken during the 1993 studies from superficial and visceral (deep) lymphocentres and cultured separately. The findings from two possums, 150S and D2033, each of which had a single gross lesion in a superficial lymphocentre were investigated in that way, and are presented here.

150S, a mature female with pouch young, had a single gross lesion in the left superficial axillary lymph node which yielded *M. bovis* and microscopic granulomatous lesions without AFOs in three lung lobes. Histology of the liver, lung, right anterior mediastinal lymph node, kidneys, adrenals, spleen, bone marrow and tonsils revealed no lesions. *Mycobacterium bovis* was recovered both from a sample of pooled deep lymph nodes (mesenteric, hepatic, left anterior mediastinal) and a pooled sample of the remaining superficial lymph nodes, indicating widespread infection.

D2033, an immature male about 15 months old, had a culture positive single gross lesion in the left superficial axillary lymph node. Histopathological examinations of liver, lung, left anterior mediastinal lymph node, kidneys, adrenals, spleen and tonsils were negative. However, *M. bovis* was recovered from a sample of pooled deep lymph nodes, indicating that there were lesions at more than one site.

Lesion distributions in tonsils, deep cervical lymph nodes and gastric and mesenteric lymphocentres (non-terminally ill possums only)

Tonsils: All 19 possums with microscopic lesions in either or both palatine tonsils had lung lesions and evidence of generalised infection. All but one possum in this group had lesions in the mesenteric lymphocentre.

Deep cervical lymph nodes: All 25 possums with tuberculous lesions in either or both deep cervical lymph nodes had lung lesions and evidence of generalised infection. Twelve of the 25 had tonsillar lesions.

Mesenteric lymphocentres: Of 43 possums with evidence of infection in the mesenteric lymphocentres, 41 also had lung lesions, 37 had liver lesions and 16 had lesions in the gastric lymphocentres.

Gastric lymphocentres: All 17 possums with tuberculous lesions in the gastric lymphocentres had lung lesions and evidence of generalised infection. Fifteen animals from this group had lesions in either tonsils or deep cervical lymph nodes.

Anterior mediastinal lymphocentre: Of 62 possums with lung lesions, 53 had accompanying lesions in either or both anterior mediastinal lymph nodes.

DISCUSSION

The findings reported in this series of papers present the most comprehensive description of the nature of naturally occurring tuberculosis in possums and allow inferences to be made about the routes of infection and excretion of the disease.

Excretion of mycobacteria is possible from both lung and kidneys, where lesions occurred commonly. Supportive evidence for a high prevalence of excretion from lung came from culture results, where 10 (36%) of 28 tracheal washings were culturally positive. These findings are supported by histopathology of lung from tuberculous possums (Cooke *et al.*, submitted), which commonly revealed free and intracellular mycobacteria in airways. The lung lesions were often extensive, frequently comprised multiple lesions at varying stages of development and size, and protruded into air spaces, providing opportunity for excretion via airways. Excretion via the respiratory route has an added potential to contaminate saliva when organisms are deposited in the pharynx as a result of normal mucus clearance mechanisms. One terminally ill possum had histopathological evidence of urinary excretion (Cooke *et al.*, submitted) but only 2% of 41 urine samples were positive and this (single) isolate was also from a terminally-ill animal. Similarly, three possums showed histopathological evidence of intestinal infection (Cooke *et al.*, submitted), but *M. bovis* was recovered from only 2% of 41 faecal samples, again from a terminally ill animal

(although, as with urine, the higher concentrations of CPC used for de-contamination would have caused greater losses of viable organisms than for tracheal washings).

Lesions were found in mammary glands of 12% of all tuberculous females examined (Jackson *et al.*, submitted) and the risk to pouch young for infection by this route is rated high. Pouch young are at risk from infected milk during their continuous attachment to the teat for about 70 days, followed by regular nursing during the remaining time to weaning at about 190 days. Furthermore, during rearing, the pouch is regularly cleaned and the pouch young is diligently groomed by its mother. Grooming continues for "back-riders" for several more months until independence. Our recovery of *M. bovis* from a pouch young about 70 days old with a terminally ill mother supports the concept of pseudovertical transmission of infection. Two other possums, still dependent on their mothers, showed microscopic lesions in lung and anterior mediastinal lymph nodes. One of these also had a smaller lesion in a deep axillary lymph node in which no acid fast organisms were seen. Pfeiffer (1994)and Morris *et al.* (1994) have both hypothesised on epidemiological grounds that this route of transmission is a key factor in maintenance of infection within local populations and in more distant spread of infection to new areas through dispersal of infected immature animals (principally males). Our findings would support a conclusion that no other demographic group of possums is exposed to infection for as long or from as many potential routes of transmission as are pouch young.

Excretion of organisms also occurs through fistulae which discharge infective material from lesions to the exterior. Possums are fastidious groomers and spend about 11% of their time outside of their dens in self-grooming activity (MacLennan, 1984). Biggins (1984) has described the methods of face washing and grooming used by brushtail possums and drew attention to the extensive use of saliva in both activities. The external openings and areas surrounding fistulae are well groomed in all affected possums other than animals at the terminally ill stage, when normal behaviour patterns are altered. Thus the oral cavity of a possum with a discharging fistula would almost certainly become contaminated during self grooming activities. All but one of the 22 possums with discharging fistulae had lesions in their mesenteric lymphocentre, eight had lesions in their gastric lymphocentre, and all 22 had evidence of generalised infection. Although it was not possible to determine the sequence of lesion development in our studies, it appears likely that possums with discharging fistulae represent a relatively advanced stage of the disease process. Thus while self-grooming can only initiate infection in exceptional situations where an infective dose derived from another animal contaminates fur and is ingested, self-grooming may

increase the number of lesion sites in infected animals. Mutual grooming could potentially allow transmission to take place from an infected animal to another but it probably occurs rarely outside mother-offspring relationships. In the Castlepoint longitudinal study (Pfeiffer and Morris, 1991), there has been no specific evidence to support it, although it is extremely difficult to assess. At other sites in both New Zealand (Fairweather *et al.*, 1987) and Australia (Winter, 1976), circumstances such as simultaneous den-sharing may be more conducive to it. In the context of this study, its overall importance remains unknown since it cannot be distinguished from other mechanisms.

Primary tuberculosis lesion complexes are well recognised in man and cattle and their location allows inferences to be made about routes of infection for these particular hosts. The pattern of distribution of lesions in our small group of possums which were judged to be in the early stages of disease shows too much variation and inconsistency to allow confident recognition of primary complexes. The single lesion in a mesenteric lymph node recorded in one possum is consistent with an alimentary route of infection. The distribution of lesions in the immature possums still dependent on their mothers is consistent with a respiratory route of infection. We are unable to offer satisfactory pathogenetic explanations for our common finding of involvement of superficial lymph nodes in presumed early cases, or the overall high frequency of occurrence of superficial lymph node lesions (94.5%) in all tuberculous possums (Jackson *et al.*, submitted). Interpretation is further clouded by apparent preferential establishment of lesions in left superficial axillary lymph nodes and a significant preference for the left deep axillary lymphocentre over the right deep axillary lymphocentre (Jackson *et al.*, submitted).

Studies of the lymphatic system of the possum (Jackson and Morris, submitted) indicate that the superficial axillary lymph nodes in the brushtail possum receive afferent vessels from the foreleg and sternal regions. The inguinal lymphocentres drain the hindlegs and tail. The deep axillary lymphocentre drains the dorsolateral aspects of the neck and thorax and receives afferent vessels from the superficial axillary lymphocentres. An efferent vessel (the inguino-axillary trunk) from the inguinal lymphocentre passes directly to the deep axillary lymphocentre which then connects with the venous system. Given this pattern of lymphatic drainage, and in the light of results from experimental infections where subcutaneous or intramuscular injections of *M. bovis* organisms consistently produced local discharging skin lesions, tuberculous lesions in superficial lymph nodes might be considered to be indicative of entry of infection through the skin. However, in all of our studies, primary tuberculous lesions have never been detected in skin, and it is difficult

to present any plausible explanation for the occurrence of lesions confined to superficial lymph nodes. Apart from discharging sinuses, open skin wounds were not seen, although a few possums had healed lacerations on ears. Haematogenous, or a mixture of haematogenous and lymphatic, spread from more distant sites to superficial lymph nodes therefore seems more likely in view of the possum's limited ability to wall off lesions. Support for both methods of spread also comes from microscopic lesion distributions within lymph nodes (Cooke *et al.*, submitted).

How efficiently possum lymph nodes filter and retain mycobacteria carried by afferent lymph vessels remains to be determined and the generally held concept of primary site infection with localisation in a regional lymph node draining the site may not apply to possums. There are sound reasons for caution before applying eutherian principles to marsupials, particularly in light of the significant differences between the anatomy of the lymphatic systems of eutherians and marsupials studied to date, viz. the American opossums, *Didelphis azarae* and *Didelphis marsupialis* (Azzali and Di Dio, 1965), the grey kangaroo, *Macropus giganteus* (Hopwood, 1980; 1988), the koala, *Phascolarctos cinereus* (Hanger and Heath, 1991) and the brushtail possum (Jackson and Morris, submitted). Hopwood (1980) drew attention to certain peculiarities of lymph flow in kangaroos where ink-delineated vessels would sometimes by-pass or traverse only part of lymph nodes, although it is not known whether this finding also applies to possums. Caution is also recommended before applying normal anatomical principles to diseased animals which may have obstructive lesions in lymph nodes, with consequent re-routing of lymph flow. Wood (1924) ligated the inguino-axillary trunks of a didelphid opossum and successfully diverted lymph from the superficial hindlimb and tail regions to the lumbar trunks, and preliminary investigations in kangaroos by Hopwood (1988) indicated that similar effects would be likely in that species. The apparent preference for left axillary nodes is intriguing. Anatomical studies (R. Jackson, pers. obs.) indicated an asymmetry of lymphatic drainage in the thorax, and it is conceivable that drainage from the lung could reach the left deep axillary lymphocentre and not the right. However no firm evidence to support this explanation has been found.

A somewhat conjectural explanation may be that the sensitivity of detection of microscopic lesions in lungs was not sufficiently high to detect all lesions and that lung lesions occurred more frequently than were recorded in early cases. Sparse microscopic lesions in large organs are particularly difficult to find and require a large effort for detection. The lung of the juvenile, H3126, was resectioned numerous times after finding a tuberculous lesion in the right mediastinal lymph node and a characteristic microscopic lesion in the right caudal lobe. No

further lesions were found. In H3132, after finding a lesion in both the right cranial lobe and the right anterior mediastinal lymph node, further sectioning revealed one more lesion but failed to demonstrate the presence of AFOs.

Although our findings do not directly indicate routes of infection, some inferences may be made indirectly from our data on routes of excretion. It is clear that organisms are available for excretion from non-terminally ill tuberculous possums, principally from lung, discharging fistulae, milk and saliva. Pouch young infection is feasible by any of these routes. These excretion patterns have potential for environmental contamination and subsequent infection by the oral or alimentary route during feeding and grooming activities, but our data indicates that mesenteric lymphocentre lesions are generally secondary to and less prevalent than lung involvement. Mesenteric lymphocentre lesions are almost always associated with a relatively advanced and often generalised stage of tuberculosis, and it is likely that alimentary infection is secondary to lung infection or discharging fistulae. Although it appears likely that B6104 was infected by the alimentary route, our data suggests that this route is not a common one in possums.

Although high prevalences of lesions in superficial lymphocentres could be explained by a percutaneous route of infection, biological plausibility for that route is reduced by an absence of infected skin wounds and the marked preponderance of lesions in axillary lymph nodes over other superficial sites. If fighting or sternal gland marking initiated infection through a percutaneous route, then different lesion distributions in superficial lymph nodes could be expected between males and females, as both these activities are exhibited predominantly by adult males. Our data showed no differences in the distribution of lesions between males and females (Jackson *et al.*, submitted). Moreover, superficial lymph nodes were involved in five of the six juvenile possums with early stage disease listed in Table 6.4 prior to sexual maturity, when fighting and territorial marking behaviour normally commences. These possums were probably infected as pouch young or dependent juveniles. This mode of infection is probably the most common for juvenile possums, while older animals may become infected by the respiratory route during periods of close contact such as agonistic encounters, mating or concurrent den sharing activities, and less frequently by the oral route following territorial marking or self grooming after occupying a contaminated den.

The pathological data from our cross-sectional studies provide as yet inconclusive explanations of routes of infection. More information may come from obtaining further early cases of disease, but they are difficult and expensive to find. In experimental settings (O'Hara *et al.*, 1976; Corner and Presidente, 1981; Buddle *et al.*, 1994), it has been shown that possums may be infected naturally from animals caged separately, but in the same room. Possums infected in this manner may be useful for studies designed to monitor the progress of natural disease and establish predilection sites. More investigations of these issues are now warranted if more definitive explanations are to be produced . Similarly, a better understanding of the pathogenesis of tuberculosis in possums will also come as our knowledge of details of the lymphatic pathways and the immunological response mechanisms of the possum improves.

Conclusions about routes of infection of tuberculosis in domestic and wild animals are necessarily derived largely from indirect sources of evidence. The high prevalence of lung lesions indicates that organisms are potentially available from lesions in the respiratory system in aerosol form and in saliva, and experimental studies have indicated that possums may readily become infected by the respiratory route. Although a process of elimination focuses attention on the respiratory route of infection as a major mechanism of transmission between adults, our pathological data does not clearly or directly implicate this route. Nevertheless, our data does strongly suggests that the respiratory route is the major excretion route in adults and the inference from this is that it is also the principal route of initiation of infection in these possums.

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CHAPTER 7

Serological tests for the diagnosis of tuberculosis in possums

Evaluation of three immunosorbent assays

Appearances to the mind are of four kinds.
Things either are what they appear to be:
or they neither are, nor appear to be:
or they are, and do not appear to be:
or they are not, yet appear to be.
Rightly to aim in all these cases is the wise man's task.
Epictetus 2nd century AD

ABSTRACT

None of the assays reliably detected possums infected with tuberculosis and they therefore have very limited value for epidemiological studies. Poor test performance was exacerbated by inconsistency between results from serially collected samples from known tuberculous possums.

Although the CF assay appeared to support an association between advanced disease states and humoral immunity, the BLOCK assay performed with equal efficiency across all stages of the disease as measured by the number of lesion sites per individual. The association between test response and stage of disease was inconclusive for the MPB70 assay.

Inconsistency between readings from assays conducted at different times and non-normally distributed data created problems for some analyses. Receiver operating characteristic analysis was the preferred method for selecting cutoff points. Pseudo-retrospective sampling gave results which were comparable to those achieved with naturalistic sampling.

INTRODUCTION

A reliable serological test which could be used to detect tuberculosis in possums would be welcomed by epidemiologists and disease control personnel. Epidemiological studies of tuberculosis in possums are hampered by the lack of a suitable test with an ability to 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 post mortem examinations and the laboratory procedures are time consuming and expensive with up to eight weeks wait for results of mycobacterial culture tests to become evident.

The cost effectiveness and efficiency of pest control operations would be considerably enhanced if disease status of possum populations and locations of foci of infected possums could be predetermined by use of an automated and inexpensive serological test.

In man and other species, serological tests which rely on detection of a humoral response have proved disappointing for diagnosis of tuberculosis. The immune response to tuberculosis is largely cell mediated with a minor humoral component (Thorns and Morris 1983). 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.*, 1991). Unfortunately, all of the tests dependent on humoral 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 anergic to the intradermal test on the premise that circulating antibody may be more detectable in cases of advanced disease.

During a five year longitudinal study of tuberculosis in possums at Castlepoint in the Wairarapa (Pfeiffer 1994), which had as one of its aims the development of a serological test, sequential serum samples were collected from possums at monthly capture-mark-release occasions. Sera were also collected, whenever possible, during pathogenesis studies in which captured possums were subjected to detailed necropsy procedures (Jackson *et al.*, submitted). All sera were stored for later use in evaluation of serological tests which could be used to determine infections status

and time of initial infection in possums studied at Castlepoint.

Three immunosorbent assays for tuberculosis in possums were recently evaluated (Buddle *et al.*, in press) 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 tested by Buddle *et al.* (in press), were used for evaluation. The preferred tests were then applied to sera from blood samples collected between April 1989 and June 1992 at monthly trapping visits in the Castlepoint longitudinal study.

MATERIALS AND METHODS

Collection of blood samples

Blood samples were collected at slaughter whenever possible from possums necropsied during a series of cross-sectional studies (see Table 7.1) conducted during investigations of the pathogenesis and prevalence of tuberculosis (Coleman *et al.*, 1994; Jackson *et al.*,[a] submitted). Necropsies were designated detailed if the diagnostic criteria included culture of lesions for *M. bovis* and/or extensive histopathological examination of multiple organs or culture of pooled lymph nodes. Necropsies were designated gross when no laboratory procedures were done. Detailed necropsies were carried out on all animals in which visible lesions of tuberculosis were detected. Sera were removed from clotted bloods by centrifugation and stored at -20°C until they could be transported to Massey University where they were later transferred to -80°C storage. Sera (negative sera), collected from 100 possums in Northland, a non-endemic region of New Zealand, where the disease has not been diagnosed in possums were stored at -20°C. All tests were performed at the Agresearch Central Veterinary Laboratory at Wallaceville using the procedures described by Buddle *et al.*, (in press).

Table 7.1. Cross-sectional study sera tested. Table showing numbers of sera from possums with positive diagnoses of tuberculosis and the origins of 251 sera classified by study location and diagnostic criteria used for postmortem examination

Study	Number with Tb	Detailed necropsies	Gross necropsy only	Total necropsied
Waio July 1993	1	13	71	84
Waio Sept 1993	3	10	47	57
Flagstaff Aug 1992	34	45	14	59
Flagstaff Aug 93	7	51	0	51
Totals	45	119	132	251

Evaluation

Culture filtrate and MPB70 test results were expressed as "absorbance indexes", 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 expressed according to the equation used by Buddle *et al.*(in press):

$$\textit{Percentage inhibition of the monoclonal antibody used} = 1 - \frac{\textit{Block assay test result } x\ 100}{\textit{negative reference serum test result}}$$

For evaluation of the assays, the diagnostic criterion or "gold standard" applied was detailed necropsy. Sera from Northland and the 1992 Flagstaff study were tested using monoclonal antibody blocking (BLOCK), *M.bovis* culture filtrate (CF), and MPB70 ELISA tests. Sera from cross-sectional studies after 1992 were tested only with the CF and MPB70 ELISAs. Because separately prepared conjugate was used for the latter series of tests, results from that series were adjusted by the difference in the means in the two tests of the negative sera recorded with the different conjugates. Persons who performed the tests were unaware of disease status of individual sera.

Because the culture filtrate and MPB70 absorbance indexes were not normally distributed, they were logtransformed to $\log_{10}$ values for all calculations and analyses which required normally distributed values.

Tests were assessed using two methods to allow a comparison of the techniques to be done. The first method used negative and positive sera from separate populations (pseudo-retrospective sampling) (Kraemer 1992). The second method used naturalistic sampling and utilised sera from animals to which the gold standard of detailed necropsy had been applied. For the pseudo-retrospective sampling method the cutoff points were determined according to the formula:

$$\text{Cutoff point} = \text{Mean } (\bar{X}) + 2.57 \text{ standard deviation (s)}$$

This point corresponds to the point which separates the top 0.5% of values and should theoretically give a cutoff value consistent with 0.995 specificity.

In the second method, the cutoff points were determined using receiver operating characteristic (ROC) curves and the cutoff point was taken as the index value immediately preceding the one associated with a decrease in specificity from 1.0.

Data were stored in Paradox for Windows Version 4.5 and Quattro Pro for Windows Version 5 (Borland International). Test evaluations were performed in Quattro Pro using Testview, Version 1.05 (Ian A. Gardner and John C. Holmes, Department of Epidemiology and Preventative Medicine, School of Veterinary Medicine, University of California, Davis, CA 35616, USA) and ROC curves were constructed using Number Cruncher Statistical System (NCSS) Version 5.03 (Dr. Jerry L. Hintze, Kaysville, Utah, USA). Other figures were constructed in Statistica for Windows (Statsoft Inc., 2325 East Street, Tulsa, USA).

The terms sensitivity, specificity, predictive values and efficiency were used as summary descriptors for evaluation of tests. Sensitivity and specificity are conditional probabilities describing test performance with reference to the diagnosis. Sensitivity is defined as the probability of having a positive test among those animals which were diagnosed as having the disease. Specificity is defined as the probability of having a negative test in those animals which do not have the disease. The predictive values are conditional probabilities describing the performance of the diagnosis with reference to the test. The predictive value of a positive test (PVP) is the probability of having a positive diagnosis in animals which have a positive test result. The predictive value of a negative test (PVN) is the probability of having a negative diagnosis in animals which have a negative test result. Efficiency (EFF) is the probability that test and diagnosis agree and is calculated from the sum of the true positives and true negatives divided by the number sampled.

RESULTS

Test evaluation using cutoffs derived from mean plus 2.57 x standard deviation values

Cutoff points with upper and lower 95% confidence intervals were calculated from Northland negative sera and are set out in Table 7.2. The cutoff points for the CF and MPB70 assays vary from those selected by Buddle *et al.* (in press), who used untransformed values for those calculations.

Table 7.2. Absorbance index means, standard deviations and cut-off points derived from tests on sera from a non-diseased possum population in Northland with 95% confidence intervals shown in parentheses

Assay	Mean	Standard deviation	Cutoff point.
CF	1.251 (1.207-1.296)*	0.223*	1.824 (1.777-1.871)*
MPB70	0.716 (0.673-0.759)*	0.216*	1.272 (1.229-1.315)*
BLOCK	0.098 (0.083-0.112)	0.073	0.285 (0.271-0.3)

* $\log_{10}$ transformed data

These cutoffs were then applied to detailed necropsy sera and the test and diagnosis results compared. Summary descriptors of the tests singly, and in combination, are set out in Table 7.3. Only forty-four sera were tested with the BLOCK assay and results of all tests and all combinations of tests for that subsample of the total of 119 sera are set out in Table 7.3. For some of the tests, the numbers in each marginal position of the 2 x 2 tables used for calculations were not large enough to ensure that estimates of sensitivity, specificity, predictive values and efficiency were reasonably unbiased.

Table 7.3. Summary test results from possums for which the diagnostic criterion was detailed necropsy using cutoff points calculated from tests on sera from a non-diseased possum population in Northland

Test	N	P	Q	SE*	SP*	PVP	PVN	EFF	χ^2
CF	119	0.38	0.11	0.27(0.15-0.42)	0.99(0.92-1.0)	0.92	0.69	0.71	18.4
MPB70	119	0.38	0.07	0.18(0.09-0.33)	1.0(0.94-1.0)	1.0	0.67	0.69	14.1
CF+MPB70	119	0.38	0.05	0.13(0.06-0.28)	1.0(0.94-1.0)	1.0	0.66	0.67	10.4
Block(B)	44	0.77	0.21	0.27(0.14-0.45)	1.0(0.66-1.0)	1.0	0.29	0.43	3.33
CF	44	0.77	0.21	0.27(0.14-0.45)	1.0(0.66-1.0)	1.0	0.29	0.43	3.33
MPB70(MPB)	44	0.77	0.14	0.18(0.07-0.35)	1.0(0.66-1.0)	1.0	0.26	0.36	2.04
Block+CF	44	0.77	0.11	0.15(0.06-0.32)	1.0(0.66-1.0)	1.0	0.26	0.34	1.66
Block+CF+MPB	44	0.77	0.09	0.12(0.04-0.28)	1.0(0.66-1.0)	1.0	0.25	0.32	1.29
Block+MPB	44	0.77	0.09	0.12(0.04-0.28)	1.0(0.66-1.0)	1.0	0.25	0.32	1.29

* Data in parentheses are upper and lower 95% confidence intervals
N = number of animals tested
P = prevalence
Q = level of the test = (true positives + false positives)/N
SE = sensitivity
SP = specificity
PVP = predictive value positive
PVN = predictive value negative
EFF = efficiency of the test = (true positives+true negatives)/N

Only three applications of the tests or test combinations had χ^2 values >3.84, the lowest level considered compatible with test legitimacy. The χ^2 value shown is calculated from the formula used by Kraemer (1992):

$$\chi^2 = N \times k(1,0) \times k(0,0) \text{ where } k(1,0) = \frac{PVN - (1 - P)}{P}$$

and

$$k(0,0) = \frac{PVP - P}{1 - P}$$

Prevalence and sample size strongly influence the χ^2 value derived with this formula and it can be seen that the sample size of 44 sera is clearly inadequate at that level of prevalence.

Evaluation of agreement between tests using Kappa

Agreement between the results of the CF and MPB70 assays for the whole sample of sera was moderate, based on the Kappa statistic shown in Table 7.4. By convention, values of Kappa from 0.4 to 0.6 are taken to indicate moderate agreement, 0.6 to 0.8, good agreement, and above 0.8, very strong agreement.

Table 7.4. Test agreement between CF and MPB70 tests for the sample of 119 possums using cut-off points derived from a non-diseased possum population in Northland

N	Test comparison	Kappa & level of agreement	McNemar's χ_1^2
119	MPB70 and CF	0.44 (0.23-0.65) poor to moderate	14.06*

Data in parentheses are upper and lower 95% confidence intervals
* denotes that one cell in the contingency table had a value less than 5

Evaluation of agreement between tests using Receiver Operating Characteristic curves

The relationship between sensitivity and specificity can be more effectively displayed with receiver operating characteristic (ROC) curves. In Figure 7.1, the ROC curves for the each assay are shown along with the diagonal ROC curve representing the level of discrimination expected by chance alone. The comparison of the areas under the curves, set out in Table 7.5, suggests that the BLOCK assay has the greatest discriminatory power although the standard errors are large and indicate considerable overlap if confidence intervals are fitted. From Figure 7.1, it can be seen that the BLOCK assay is the best performing test when high specificity is nominated, and that the culture filtrate test performance is intermediate, and the MPB70 assay performs poorly and approaches the random curve as specificity decreases.

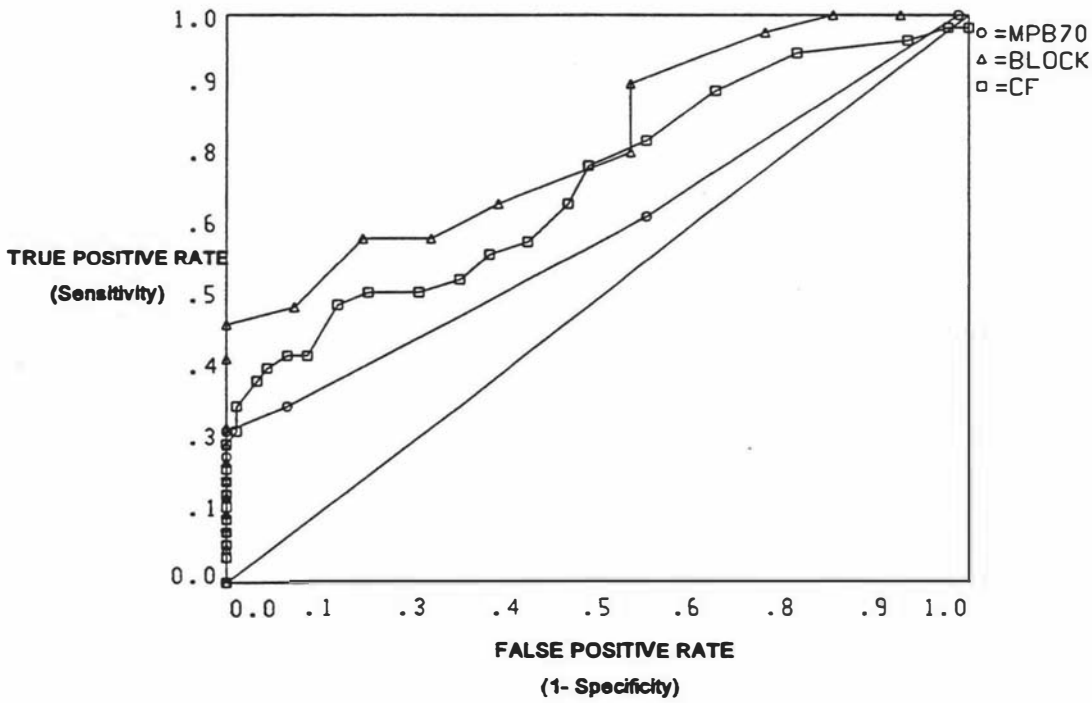


Figure 7.1. Receiver-operating characteristic curves for Culture Filtrate, BLOCK and MPB70 assays

Table 7.5. Comparison of areas under the curves for CF, MPB70 and Block assays

Assay	No. diseased	No. non-diseased	Area under curve	Standard error
CF	45	74	0.703	0.132
MPB70	45	74	0.614	0.125
Block	34	10	0.758	0.139

Cutoff points were taken from ROC curve statistics and were selected as the last absorbance index value with a corresponding specificity of 1.0, before reducing to <1.0. The cutoff points were then applied and the test statistics shown in Table 7.6 calculated. When the test statistics using cutoff points derived from ROC curves were compared with those calculated from the expression, $\overline{X} + (2.57 \times s)$, it can be seen that sensitivity for CF was unchanged, for MPB70 it was slightly increased, whereas for the BLOCK assay it was much improved both in terms of sensitivity and legitimacy as shown by an improved χ^2 value. All tests operated with equal efficiency for males and females and mature and immature animals.

Table 7.6. Summary of test results from possums for which the diagnostic criterion was detailed necropsy using cut-off points derived from ROC curves at the point of the lowest index value with a corresponding specificity equal to 1.0

Assay	Cut-off point	N	P	Q	SE	SP	PVP	PVN	EFF	χ^2
CF	1.886	119	0.38	0.1	0.27(0.15-0.42)	1.0(0.94-1.0)	1.0	0.69	0.72	21.95
MPB70	1.109	119	0.38	0.1	0.27(0.15-0.42)	1.0(0.94-1.0)	1.0	0.69	0.72	21.95
Block	0.225	44	0.77	0.34	0.44(0.28-0.62)	1.0(0.66-1.0)	1.0	0.35	0.57	6.69

Correlations between test absorbance indexes

The correlations between the test absorbance indexes of tuberculous and of non tuberculous possums were examined and the results are shown in Table 7.7. Overall, the correlations between the indexes derived from tuberculous animals were consistently higher than those derived from non-tuberculous possums. This overall difference was examined in more detail by producing scatterplots (Figures 7.2a, 7.2b and 7.2c) showing the distributions of the relationships between $\log_{10}$CF and $\log_{10}$MPB70 values .

Table 7.7. Correlations between test absorbance indexes for tuberculous and non-tuberculous possums diagnosed by detailed necropsy

Absorbance index variables	N	Disease status	r	R^2
log_{10}CF and log_{10}MPB70	45	+	+0.71	0.51
log_{10}CF and Block	34	+	+0.49	0.24
log_{10}MPB70 and Block	34	+	+0.56	0.32
log_{10}CF and log_{10}MPB70	74	-	+0.34	0.12
log_{10}CF and Block	10	-	+0.34	0.12
log_{10}MPB70 and Block	10	-	+0.02	0.00

r = correlation coefficient R^2 = coefficient of determination N = sample size

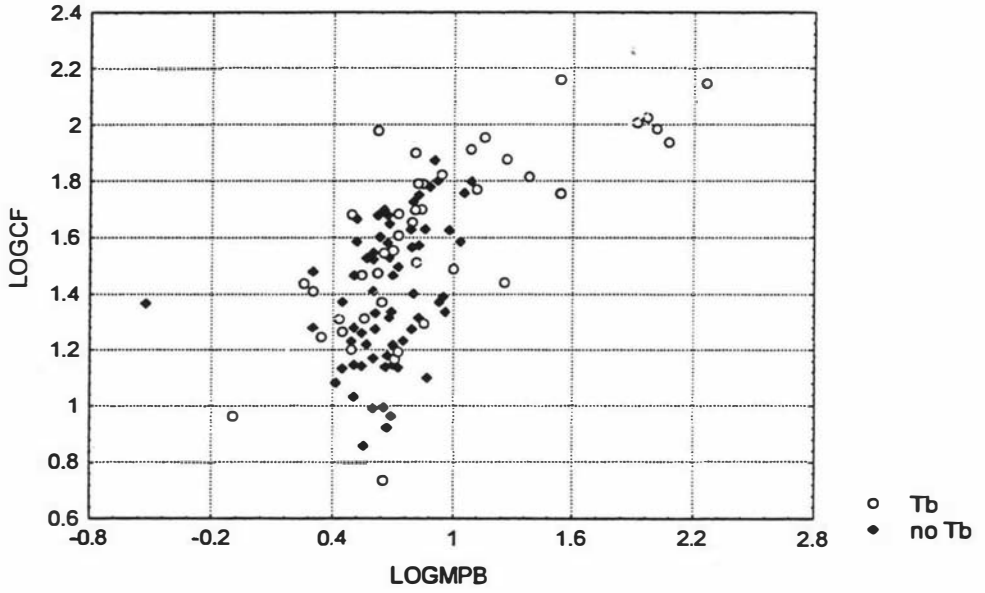


Figure 7.2a Scatter plot of log_{10}MPB70 indexes and log_{10} CF indexes of tuberculous and non-tuberculous possums

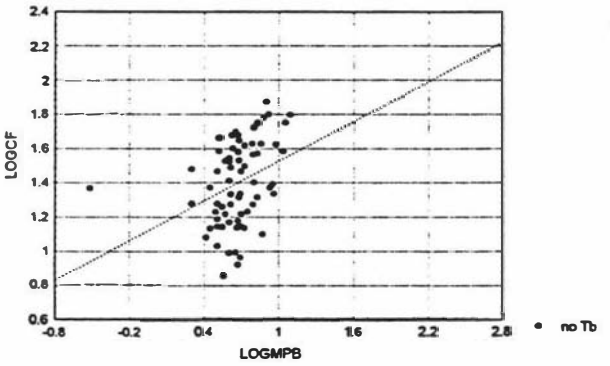


Figure 7.2b Scatterplot of log_{10}MPB70 and log_{10}CF indexes in non-tuberculous possums. r = +0.34

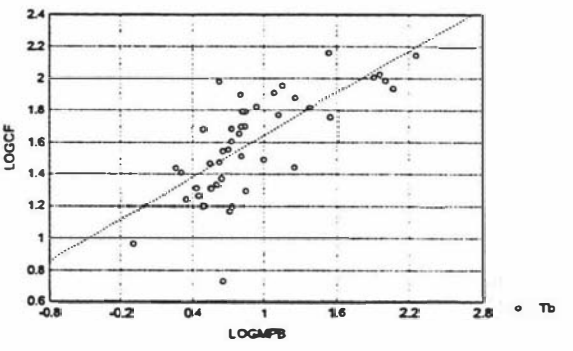


Figure 7.2c. Scatterplot of log_{10}MPB70 and log_{10}CF indexes in tuberculous possums. r = +0.71

The scatterplots illustrate the wide range of $\log_{10}$CF indexes for non-tuberculous possums occurring in relation to a narrow band of $\log_{10}$MPB70 values and illustrate the lower correlation between the indexes in non-tuberculous animals.

Lesion frequencies and test results

The numbers of lesion sites (Jackson *et al.*, submitted) containing gross and gross plus microscopic (total) lesions per individual were available for 42 sera tested by MPB70 and CF assays and the summary statistics for the frequencies of the two types of lesions are set out in Table 7.8. Similar summary statistics for 31 possums tested by the BLOCK assay are set out in Table 7.9.

Table 7.8. Summary statistics for frequency of gross and gross plus microscopic lesion sites per individual possum tested by MPB70 and CF ELISAs

Type of lesion	*Mean	s	Min	1st Q	Median	3rd Q	Max
Gross (N=42)	4.1(3.2-4.9)	2.7	0	2	4	6	10
Gross + micro(N=42)	10.4(8.4-12.4)	6.3	1	4	10.5	15	23

*Mean: values in parentheses are values of low and high 95% confidence intervals

Table 7.9. Summary statistics for frequency of gross and gross plus microscopic lesion sites per individual possum tested by the BLOCK assay

Type of lesion	*Mean	s	Min	1st Q	Median	3rd Q	Max
Gross (N=31)	3.9(2.9-4.8)	2.58	0	1	4	6	9
Gross + micro(N=31)	10.9(8.6-13.2)	6.34	6.34	4	10.5	15	23

*Mean: values in parentheses are values of low and high 95% confidence intervals

The nonparametric Wilcoxon rank-sum test was used to test the equality of distributions of gross and total lesion sites in test positive and test negative tuberculous possums. The results are shown in Table 7.10.

Table 7.10. Results from Wilcoxon rank-sum tests of equality of medians of numbers of gross and numbers of gross plus microscopic lesion sites in tuberculous possums categorised by positive and negative test results

ELISA	Lesion type	Test negatives	Test positives	p value
MPB70	gross	30	12	0.08
BLOCK	gross	17	14	0.87
CF	gross	30	12	0.001
MPB70	gross + microscopic	30	12	0.03
BLOCK	gross + microscopic	17	14	0.33
CF	gross + microscopic	30	12	0.001

p value is test of equality of mean of ranks of numbers of lesions per individual

No significant differences were shown with the Block assay over the ranges of gross and total lesion sites. This finding contrasted with the highly significant differences shown for gross and total lesion site distributions for the CF assay, total lesion distributions for the MPB70 assay and the results strongly suggested ($p = 0.08$) a difference in gross lesion distributions for the MPB70 assay. The distributions of numbers of lesion sites for the various test positive and test negatives are illustrated in histograms in Figures 7.3 to 7.8.

These analyses show that tuberculous possums which test positive to the MPB70 and CF ELISAs are more likely to have more lesion sites affected than those which have negative tests, and of the two assays the effect is most marked with the CF assay. In contrast, the Block assay response is independent of the number of lesion sites affected, and positive and negative tests occur equally over the full range of numbers of lesion sites.

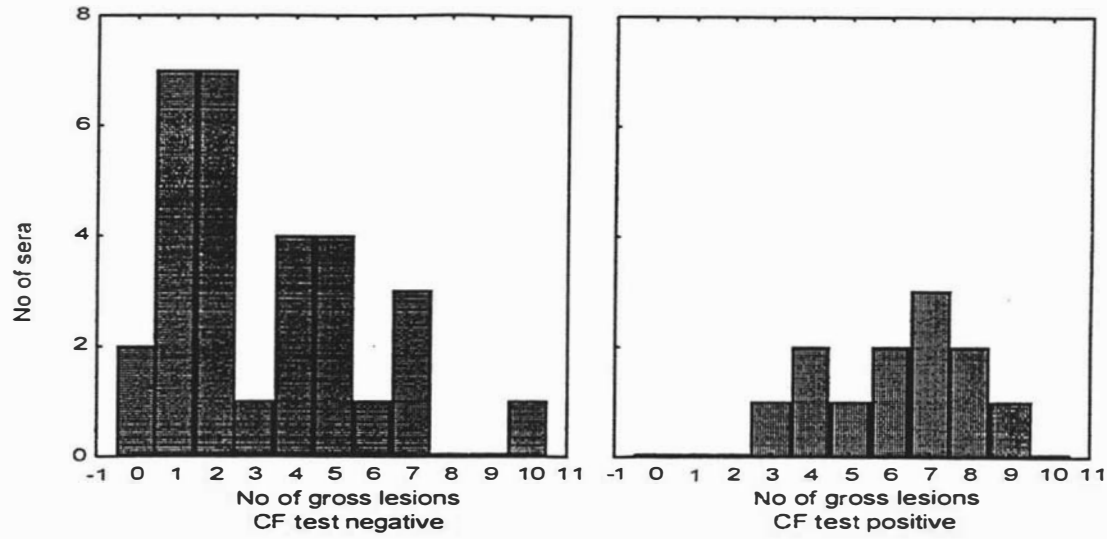


Figure 7.3. Histograms showing frequencies of positive and negative CF ELISA sera categorised by number of gross lesion sites per individual

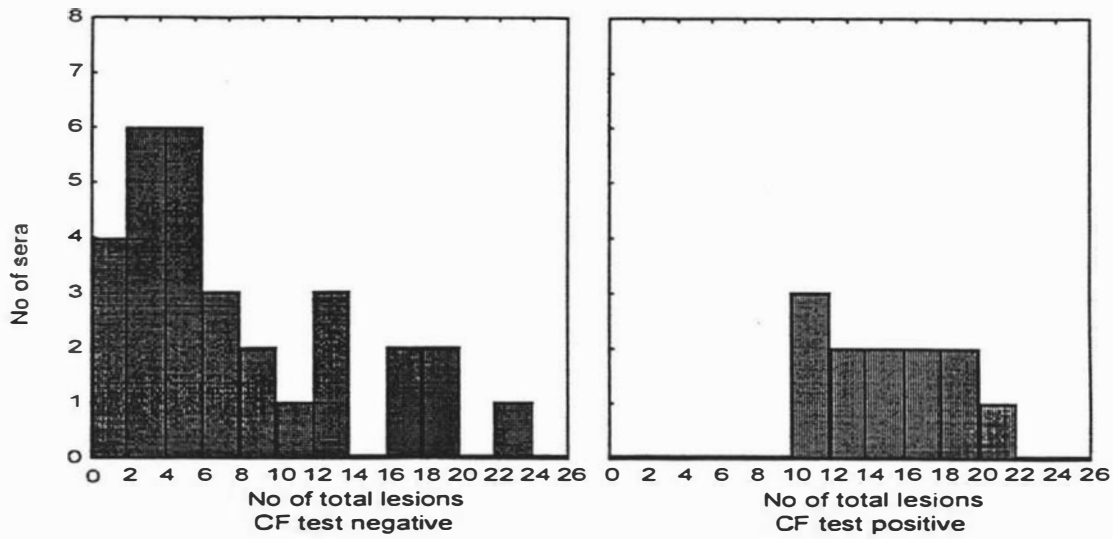


Figure 7.4. Histograms showing frequencies of negative and positive CF ELISA sera categorised by the number of gross plus microscopic (total) lesion sites per individual

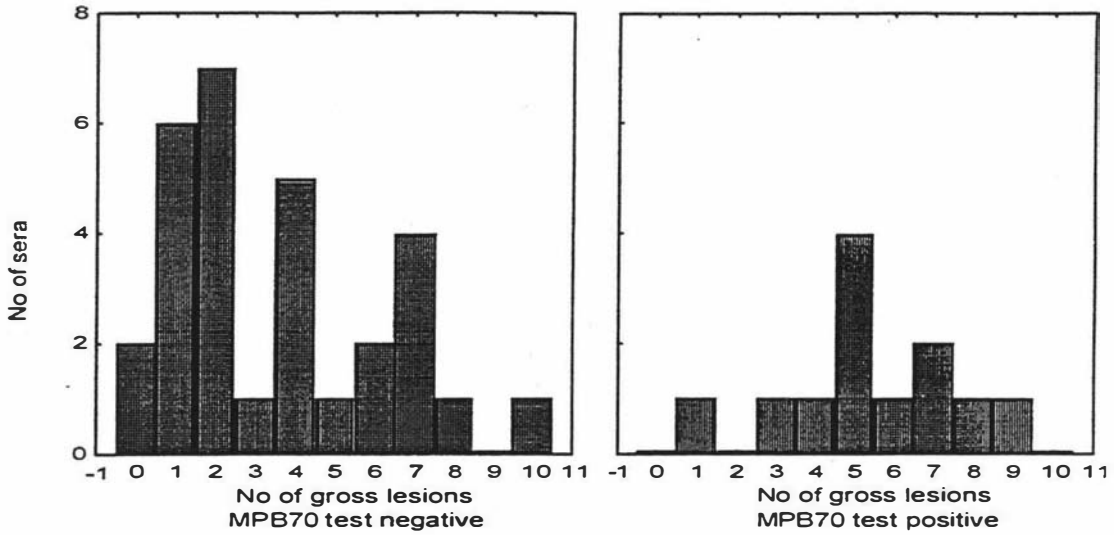


Figure 7.5. Histograms showing frequencies of positive and negative MPB70 ELISA sera categorised by the number of gross lesions sites per individual

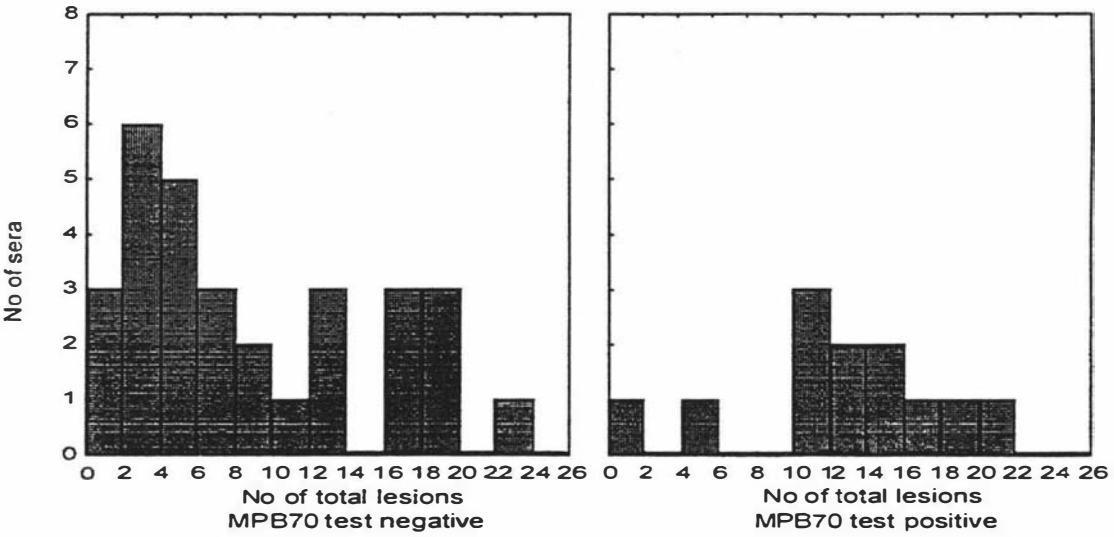


Figure 7.6. Histograms of frequencies of negative and positive MPB70 ELISA sera categorised by the number of total lesion sites per individual

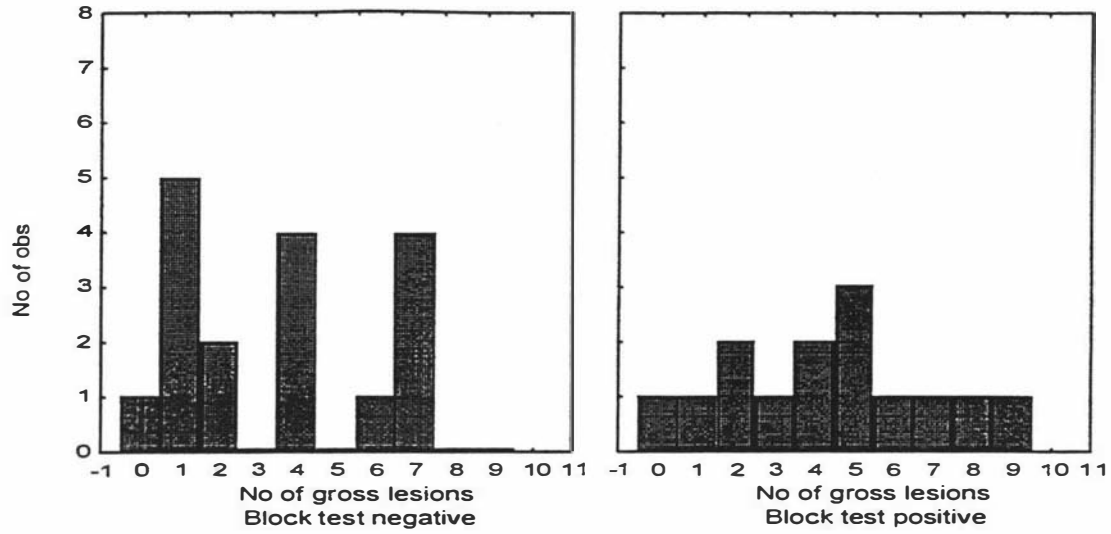


Figure 7.7. Histograms showing frequencies of positive and negative Block assay sera categorised by the number of gross lesions per individual.

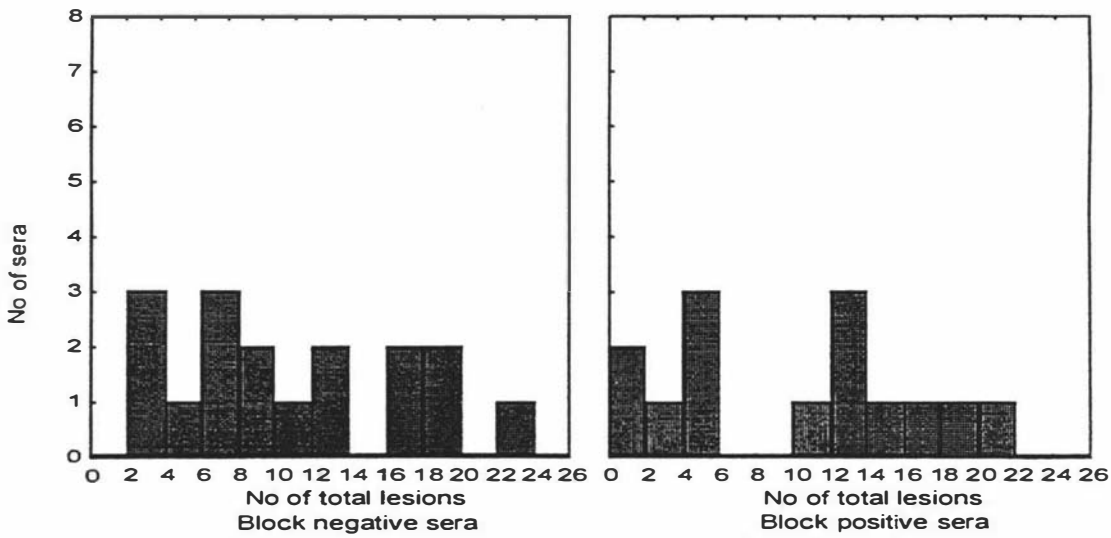


Figure 7.8. Histograms of frequencies of positive and negative Block assay sera categorised by the number of total lesions per individual

Application of the BLOCK assay to sera from the Castlepoint longitudinal study

The BLOCK assay, using the cutoff point derived from ROC analysis, was used to test 1520 sera collected at the first 32 monthly trapping occasions. The sera had been collected from 366 possums and the number of samples per possum varied from one to 19.

Tuberculosis had been confirmed in 34 of these animals by culture or histology following either clinical examination at trapping visits or at necropsy. The BLOCK assay identified nine of those 34 possums by 13 positive tests from 61 separate serum samples. The testing histories of those nine animals are set out in Table 7.11. Of 13 test positive sera, only 4 tested positive prior to time of clinical diagnosis. Serial absorbance index values from individuals varied markedly between collection occasions and showed no regular temporal patterns.

Twenty-six of 124 serum samples from 20 other possums returned positive tests. The number of sera tested per possum in this group varied from one to 13.

Of those 20 test positive animals, nine were necropsy and culture test negative in late 1994, when the study site was depopulated (Lugton pers comm). Eight were lost to follow-up, one died and was not necropsied, one had no gross lesions at necropsy and the remaining animal was both necropsy and culture test negative. The mother of one possum lost to follow-up was tuberculous at the time the young possum was still *in marsupio*. This offspring tested negative at independence but was positive two months later before becoming lost. This was the only animal with a history which suggested a high probability of infection.

Fourteen of the 20 test positive possums were test positive on single occasions and each of the remaining six returned two positive tests.

Table 7.11. Testing histories of nine tuberculous possums which were identified as positive by the BLOCK assay

MONTHS	D2398	D3513	D3620	D3644	D3674	D3680	D3709	D3719	D3735
1	Tb+D	-	-	Tb-	Tb+D	-	-	-	-
2									
3		-		Tb+		-	-	-	-
4									
5		Tb-	-	Tb-		+	-	-	-
6									
7		Tb-	-	Tb-		-	-	-	-
8									
9		Tb-	-			+	-	-	-
10				Tb+					
11		Tb+	Tb+			-		-	-
12			D	Tb+					
13		D				-	-	-	Tb-
14				D					
15						-		-	Tb-
16							-		
17						-		-	Tb+
18						Tb+	-		D
19								-	
20						D			
21								-	
22							-		
23								-	
24							+		
25									
26									
27							TbD	+	
28									
29								Tb	

D3719 died 2 months after Tb was diagnosed
D = Dead
Tb = confirmed clinical diagnosis
+ = positive serology
- = negative serology

Table 7.11. Testing histories of nine tuberculous possums which were identified as positive by the BLOCK assay

MONTHS	D2398	D3513	D3620	D3644	D3674	D3680	D3709	D3719	D3735
1	Tb☹✝	☺	☺	Tb☺	Tb☹✝	☺	☺	☺	☺
2									
3		☺		Tb☹		☺	☺	☺	☺
4									
5		Tb☺	☺	Tb☺		☹	☺	☺	☺
6									
7		Tb☺	☺	Tb☺		☺	☺	☺	☺
8									
9		Tb☺	☺			☹	☺	☺	☺
10				Tb☹					
11		Tb☹	Tb☹			☺		☺	☺
12			✝	Tb☹					
13		✝				☺	☺	☺	Tb☺
14				✝					
15						☺		☺	Tb☺
16							☺		
17						☺		☺	Tb☹
18						Tb☹	☺		✝
19								☺	
20						✝			
21								☺	
22							☺		
23								☺	
24							☹		
25									
26									
27							Tb✝	☹	
28									
29								Tb	

D3719 died 2 months after Tb was diagnosed
✝ = dead
Tb = confirmed clinical diagnosis
☹ = positive serology
☺ = negative serology

Application of the MPB70 assay to sera from the Castlepoint longitudinal study

When the cutoff point derived from the ROC curve was applied to the longitudinal study data, several hundred sera showed positive tests, and it was immediately obvious that the cutoff point was set too low. The longitudinal study sera had been processed separately and when all indexes were examined it was found that the index means (expressed in raw data units, but derived from $\log_{10}$ transformed data) were 1.056 for 1307 longitudinal sera, 0.824 for 44 sera from Flagstaff 1992, and 0.86 for 75 sera from other cross-sectional studies. Means for negative sera tested at the same times as Flagstaff 1992 and the other 75 sera were 0.716 and 0.858 respectively. Consequently, because of the large disparity between means, no predetermined cutoff could reasonably be applied and a substitute value which selected 29 possums as positives (i.e. at a similar level to the BLOCK assay) from the high end of the range of values was chosen.

The MPB70 ELISA was applied to 1307 sera collected during the first 35 months of the longitudinal study. The sera had been collected from 332 possums and the number of samples per possums varied from one to 15. Tuberculosis was confirmed in 29 of these animals by culture or histology following either clinical examination at trapping visits or at necropsy.

Eight of those 29 possums were identified by nine positive tests from 44 separate serum samples from those particular possums. The testing histories of these eight confirmed tuberculous animals are set out in Table 7.12. Of 9 test positive sera, only 2 tested positive prior to time of confirmed diagnosis. As with the BLOCK assay, indexes varied markedly between occasions and showed no regular temporal patterns. Three possums tested positive to both BLOCK and MPB70 tests.

Thirty-seven of 99 serum samples from the remaining 21 possums returned positive tests. The number of sera tested per possum in this group varied from one to 9. Of this group of 21 test positive animals, eight were necropsy plus culture or histopathology test negative, three had no gross lesions at necropsy, two were found in an autolysed state and eight were lost to follow-up. None in this group tested positive to both assays and none had a history suggestive of tuberculosis.

Seventeen of the 21 test positive group were test positive on single occasions, three on two occasions, one on five and the other on nine occasions. No gross or microscopic lesions were seen in this latter animal at postmortem examinations.

Table 7.12. Testing histories of eight confirmed tuberculous possums which were identified as positive by the MPB70 ELISA

MONTHS	D2906	D2950	D3513	D3542	D3598	D3719	D3729	D3735
1	☺	Tb☹✝	☺	Tb☹	Tb☹	☺	☺	☺
2				✝				
3	☺		Tb☺		✝			☺
4								
5	Tb☺		Tb☹			☺	☺	
6	✝							
7			Tb☺			☺	☺	☺
8								
9			Tb☺			☺	☺	☺
10								
11			Tb*☺☺			☺	☺	☺
12								
13			✝			☺	☺	Tb☺
14								
15						☺	☺	
16								
17						☺		Tb*☹☹
18							☺	✝
19						☺		
20							☹	
21						☺		
22								
23						☺	☺	
24								
25							Tb✝	
26								
27						*☹☹		
29						Tb☺		
31						✝		

✝ = dead
Tb = confirmed clinical diagnosis
☹ = positive serology
☺ = negative serology
* indicates positive to both MPB70 and BLOCK assays

DISCUSSION

Test performance was disappointingly poor and was exacerbated by inconsistency between test results from serially collected samples. This was demonstrated when the BLOCK and MPB70 assays were used on Castlepoint sera from known tuberculous possums. Overall the study clearly demonstrated that the assays could not reliably detect infection and they therefore have very limited epidemiological or practical value.

The calculated 95% confidence intervals for the specificity of the CF and MPB70 tests were 0.94 to 1.0, and for the BLOCK assay were 0.66 to 1.0. The precision of these estimates was largely influenced by the low numbers of sera available for evaluation. In the case of the BLOCK assay, the small sample size may also have influenced the results of the χ^2 test for legitimacy. However, this assay should not be ruled out on those grounds alone, because the while the χ^2 test allows an assay to be ruled in it does not allow ruling out if N is small.

All three assays had low sensitivity when cutoff points were selected to maximise specificity. Tests with low sensitivity and high specificity may cause serious under-estimates of true positives which cannot be distinguished from false positives. As prevalence decreases, the ratio of false positives to true positives increases; an effect which becomes most marked at low levels of sensitivity and low prevalence.

If the methods for calculating the cutoff points are taken into consideration, it is reasonable to expect that more precise estimates of specificity would lie closer to the upper end of their ranges of confidence intervals. However, even if that premise was true, an unacceptably high number of false positives could still have been expected when large numbers of sera were tested, and this may have happened when the assays were used on the Castlepoint sera. The methods used to comply to the "gold standard" were the best available, and although some misclassification may have occurred, it should not have seriously affected the results.

The process of evaluating the tests used both pseudo-retrospective and naturalistic sampling and allows some comparison of the two methods used to calculate cutoff points. The problems associated with working with assay readings which are not normally distributed became evident in this study and inconsistency between assays conducted at different times was also encountered.

The study also allowed a quantitative analysis of the often stated perception that humoral immunity in tuberculosis is usually restricted to advanced disease states.

These particular aspects of the study, although not of direct importance to the main issues under examination are worthy of further consideration in this discussion.

Test evaluation ideally uses naturalistic sampling which involves a diagnosis and test for all animals in the group. Although conceptually attractive, it is difficult to do 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 problems with the method are that it ideally requires not only a large total sample size and reasonably large numbers of infected and non-infected animals, but it also requires reasonably large numbers of test positive and negative samples. 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. It is an attractive method mainly because it is logistically simple and relatively cheap to implement.. It is attended by two potential problems which are relevant to veterinary investigations and which have influenced Kraemer (1992) to firmly advise against reliance on this method. The first is that two separate populations are involved, a high risk and a low risk group, neither of which may be fully representative of the population of interest. The second difficulty is that both specificity and to a lesser extent, sensitivity are both known to vary between populations.

Tables 7.3 and 7.6 show good agreement between the two sampling methods for the CF and MPB70 assays where the sample size was reasonable, but poorer agreement with the BLOCK assay which suffered from a small sample size. However, some of the difference between the levels of agreement may have been due to the use of two different methods for calculation of cutoff points, and if this is taken into consideration it would seem that the arguments against the use of pseudo-retrospective sampling may not have been justified in this study.

The use of ROC curves for evaluation of medical tests has been discussed and advocated by numerous authors including Erdreich and Lee (1981), Swets and Pickett (1982), Swets (1988), Fletcher, Fletcher and Wagner (1988), Begg (1991), and Kraemer (1992). 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 test. 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 experience from this study was that the ROC analysis was computationally easy to perform and had less attendant problems than the alternative method.

Some problems were encountered with data which was not normally distributed and required transformation before means and standard deviations, which are calculated under an assumption of normality, could be determined. Although some data can be easily transformed to conform to a normal distribution, this is not always the case, particularly when dealing with small samples. In such cases, it is often only possible to achieve a closer approximation to normality. Even if non-normally distributed data is known to have come from a larger normally distributed sample, the analysis still requires that the subsample data follows a normal distribution.

Inconsistency between results of assays conducted at different times caused some problems which could only be partly compensated for. It was possible to adjust values by differences in means when the differences were due to the use of separately prepared conjugates, although this action probably caused some loss of precision. The problem encountered with differences between the MPB70 assays conducted at different times was more difficult, and was dealt with, again probably imprecisely, by selecting the same number of possums from the high end of the range of values as were determined positive by the BLOCK test.

The Kappa statistic for agreement between the CF and the MPB70 tests on the cross-sectionally derived sera was 0.44 (c.i. 0.23 - 0.65). This statistic measures how closely tests agree after accounting for chance agreement. A Kappa of 0.44 means that 44% of the potential agreement beyond chance was actually achieved.

Although the numbers of animals involved were small there was no apparent between sex difference in test performance.

When the BLOCK and MPB70 assays were performed on the longitudinal sera, only three sera from the true positives were positive to both assays (Table 7.12). Serially collected sera from known positive animals did not always test positive and obviously the immunological processes

operating in these animals did not allow the expression of constant positive results over time. A satisfactory explanation for this is beyond the scope of this paper but the phenomonem has rarely been examined or reported in animal studies involving tuberculosis prior to this investigation and may be more common than is currently thought. In any case it certainly contributed to poor test performance and should be routinely checked for in serological studies of mycobacterial infections. Multivariable analysis designed to test for associations between physiological states and stages and aspects of the disease process may help to explain the differences over time but such analyses are computationally difficult because of the presence of time varying variables and lack of independence between serially collected data.

The BLOCK test appeared to perform with equal efficiency across all stages of disease as measured by the number of lesion sites per individual, whereas the CF assay detected advanced disease states more efficiently and the MPB70 assay appeared intermediate in that ability. Comment about the immunological basis for these results is beyond the scope of this paper, but depending on the assay used, there is both support and denial for the concept that humoral immunity mainly attends advanced disease states.

ACKNOWLEDGMENTS

Assays were carried out by staff at the Animal Research Centre at Wallaceville. They and Dr. Anne Nolan from Central Veterinary Laboratory, Addlestone, UK, were always very helpful and cheerfully gave advice and assistance whenever it was sought.

CHAPTER 8

A Longitudinal Study of Tuberculosis in Possums and Cattle

INTRODUCTION

Pfeiffer (1994) described the design and conduct of a longitudinal study of tuberculosis in a possum population in contact with beef cattle. He reported the analyses of results from April 1989 to January 1991, which represented all of Phase I and most of Phase II of the study. Responsibility for the continuing conduct of the study then passed to other investigators. The description presented here covers the period from the start in April 1989 until July 1993 when new personnel were again introduced to conduct the final stages of the study. The design and conduct of the study were unaltered but its scope was extended in Phase III by disease transmission studies. The aims of the longitudinal study were to investigate and describe the epidemiology of naturally occurring tuberculosis caused by *M. bovis* in possums, to investigate the modes of transmission of the disease from possums to cattle and to produce data and statistics for incorporation into a computer simulation model. The overall aim of the investigation was to improve control methods through a better understanding of the epidemiology of the disease.

MATERIALS AND METHODS

The study site has been described in detail by Pfeiffer (1994) but some additional observations about the site and the surrounding areas are warranted here. The 21 ha site is located in a paddock named Backdrop, the northern side of which boundaries the next property, at the head of a valley running in an east-west direction

It is part of a sheep and beef cattle farm of approximately 1000 hectares, Waio, which runs about 3,000 crossbred (Romney Marsh) ewes and replacements, about 80 crossbred beef breeding cows and 150 mixed age fattening cattle. The farm is still being developed. Extensive clearing of predominantly manuka (*Leptospermum scoparium*) scrub was undertaken in the 1970s to establish pasture land. In some of the steeper gullies, indigenous scrub and forest persist and occur in patches throughout the farm. The farm also has several 10 to 20 year old *Pinus radiata* plantations.

The eastern boundary of the farm is a road running along the sea coast, the northern boundary is another extensive steep and rugged property which is covered largely by indigenous scrub and forest. The western neighbouring farm comprises an extensive *Pinus radiata* plantation with plentiful vegetation beneath and between the young trees. The southern boundary runs beside a road running along a valley floor.

The extensive properties to the north and west of Waio provide good habitats for possums and support large populations of these animals. Wild red deer and feral pigs also inhabit these properties.

Backdrop has been partially cleared of manuka and pasture has established in the clearings and on the valley floor. The paddock has not been topdressed with fertiliser. Water is plentiful in creeks and gullies throughout most of the year except in dry summer periods.

The southern side of the site (Plate 8.2) is on the sunny side of the valley and is consequently drier than the northern shady side. The predominant vegetation on the souther and eastern side is mature manuka but some gorse grows along the southwest boundary and pasture is plentiful in small clearings. Flax is absent from the southern side.

Plate 8.1 The middle region of the northern side of the study site where possum density and tuberculosis prevalence is high

Plate 8.2. The manuka clad southern side of the study site

The predominant species on the northern side (Plate 8.1) is manuka and this wetter side carries rich and diverse vegetation including a wide variety of food trees and shrubs utilised by possums. Gorse is plentiful, particularly along the northern boundary, which is also partially marked by a row of mature *Pinus radiata* trees. Large mature flax plants are common and mingimingi (*Cyathodes juniperina*) and *Coprosma sp.* are plentiful in the understorey. Palatable plants include *Geniostoma rupestre*, Mahoe (*Melicytus ramiflous*), Lacebark (*Hoheria sexstylosa*), Rangiora (*Brachyglottis repanda*), Mamaku (*Cyathea medullaris*) and blecknum ferns. There are several small well pastured clearings throughout

A gully, known as Ponga gully, so called because of its high density of *Cyathea* species, bounds the site on the north west side and appears to carry a reasonably dense population of possums as judged from possum sign and evidence of browse. Some identified possums which are regularly caught on the site, den in Ponga gully.

Only minor alterations were made to the materials and methods described by Pfeiffer (1994) regarding the conduct of the field study. The number of trapping nights per monthly visit was reduced to three in May 1991. From January 1993 on, all sera were stored at -80°C.

Cultural examination for mycobacteria of all possums which died from causes other than tuberculosis and which on necropsy showed no gross lesions of tuberculosis, commenced in July 1990. Initially a piece of lung together with several mesenteric lymph nodes was submitted but this selection was changed in August 1992 to a pooled selection of lymph nodes which included the superficial and deep axillary lymph nodes, inguinal lymphocentres, mesenteric, gastric, hepatic, anterior mediastinal and superficial and deep cervical lymph nodes.

Data analysis

Standard Jolly-Seber mark-recapture population estimates (Seber 1982) were calculated using the JS computer program (M. Efford, Landcare Research, Private Bag 1930, Dunedin). An assumption for the calculation of population size using this method is that there is no change in population size during each trapping period. Not all of the analyses conducted by Pfeiffer (1994) were applied to the extended data set. Home range estimations, analyses involving geographical information systems and time-space clustering of the occurrence of tuberculosis in possums were not done. Time varying covariates were not included in analysis of event histories of possums and

risk factors such as weather and seasonal effects on incidence of clinical disease were not assessed. Correspondence analysis was not carried out.

Analyses, storage and linking of information were conducted in the databases EPI-INFO version 5.02 (USD Incorporated, Stone Mountain, Georgia, U.S.A.), PANACEA (Pan Livestock Services, Reading, England) and Paradox 5.0 for Windows (Borland International, Scotts Valley, California, U.S.A.) and the spreadsheet programme Quattro Pro for Windows Version 5.0 (Borland International). Repeated measures analysis of variance and survival analyses were conducted in NCSS 6.0, (Number Cruncher Statistical Systems, Dr. Jerry L. Hintze, Kaysville, Utah, U.S.A.). Statistix 4.0 (Analytical Software, 1958 Eldridge Avenue, Box 130204 St. Paul, MN, U.S.A.) was used for logistic regression.

Den site locations were recorded in the geographical information system (GIS) PC-Arc/Info version 3.4 (Environmental Systems Research Institute, Redlands, California, U.S.A.) which was also used to produce a digital terrain model of the study site. Maps showing den sites and trap locations were generated in ArcView (Environmental Systems Research Institute) and edited in Word Perfect Draw which was also used extensively to produce charts.

POSSUM ECOLOGY

Trapping statistics

During the first 52 months of the longitudinal study, traps were set on 55,667 occasions over 206 trap nights during 52 monthly visits. Traps were set for five consecutive nights at each trapping visit up to April 1991, after which the number of trap nights per month was reduced to three. This step was taken for logistic and financial reasons after examination of trapping statistics for the period from the 13th to the 25th month had shown that 86.6% of known possums and 70.2% of previously unknown possums were captured in the first three trapping nights.

The number of traps set and the proportion which caught possums each month are shown in Figure 8.1. The average catch success was 0.248.

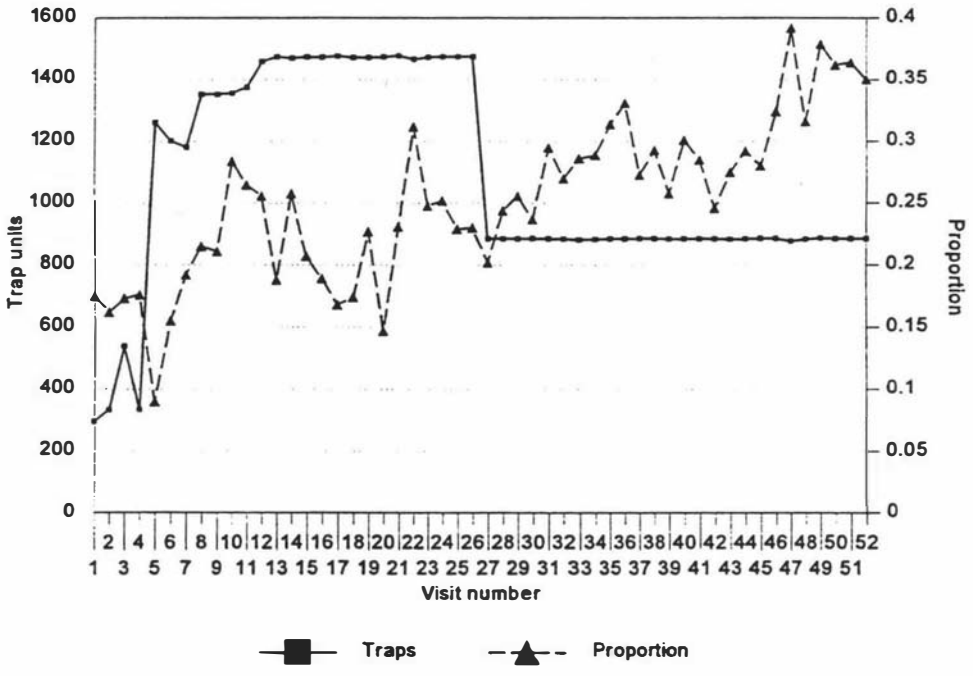


Figure 8.1. Trapcatch statistics

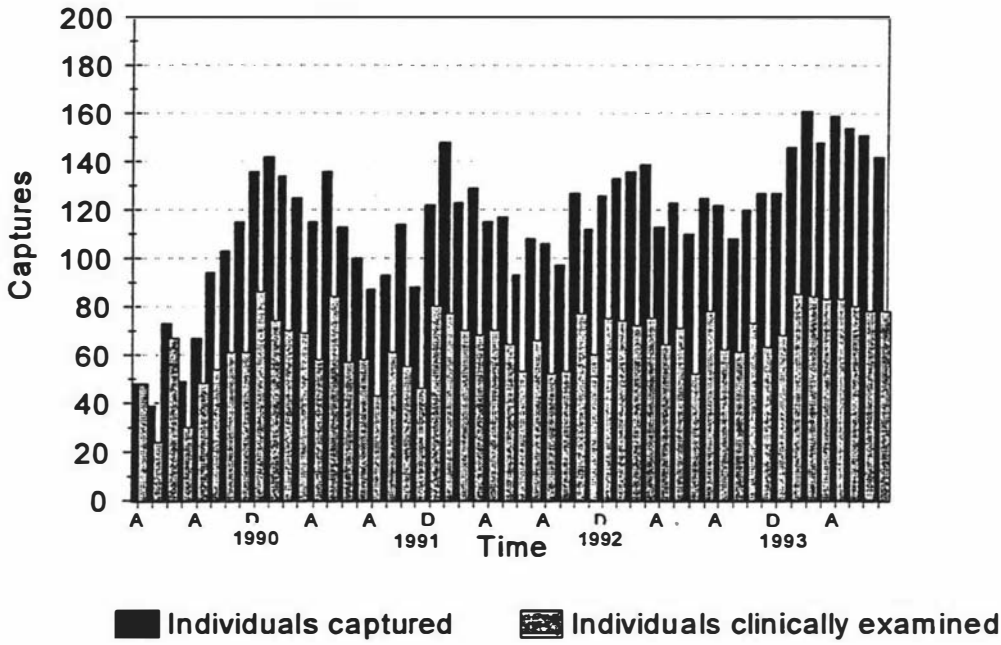


Figure 8.2. Individuals captured and individuals clinically examined at monthly visits

The number of possums anaesthetised and clinically examined at each visit ranged from 24 to 85 with a mean of 65 (Figure 8.2). A total of 632 possums were captured and individually identified and a further ten previously unidentified animals were found dead on the study site. Of that total, 243 (38.45%) were female, 389 (61.56%) were male, and for the remaining 10 (1.58%), sex was not recorded.

The number of possums caught in each February to January 12 month period from 1990 to 1993 classified by age and sex are shown in Table 8.1. Females were classified as immature if their pouch was undeveloped and did not contain a pouch young. Males were classified as immature if testicle width at the point of greatest circumference in the horizontal axis was less than or equal to 14 mm.

Table 8.1. Number of possums caught in yearly time periods between February 1990 and January 1993 classified by sex and maturity

Time period	Total examined	MM	MI	FM	FI
Feb 1990 to Jan 1991	242	97	43	81	21
Feb 1991 to Jan 1992	274	119	57	65	33
Feb 1992 to Jan 1993	258	108	52	69	29

MI = male immature FI = female immature
MM = male mature FM= female mature

There were no statistically significant between year differences in the proportions of immatures to matures for either sex (χ^2 for linear trend for males = 0.01, p = 0.9, and for females = 2.06, p = 0.15).

Estimates of age for 188 of 204 possums (82 females and 106 males) known to have died during the period under consideration were made from body size and testicle width measurements (N= 110), tooth cementum layers (N= 40) and from tooth wear records (N= 38). The average age at death for all possums was 23 months (mean = 23 months, median = 16, N = 188). The average age at death for males (mean = 19 months, median = 14, N = 106) was less than for females (mean = 29 months, median = 19, N = 82). These data are positively skewed and the relative distributions of age at death expressed to the nearest half year are shown in Figure 8.3.

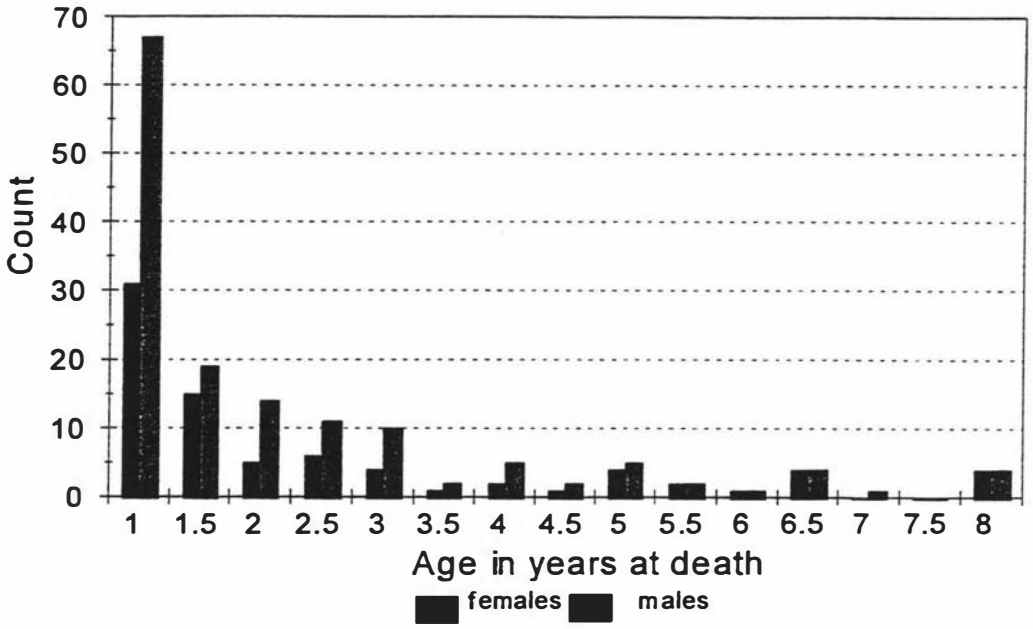


Figure 8.3. Relative frequency of ages of female and male possums for which death was recorded.

Reproduction

Reproduction showed a regular between year cyclical pattern (Figure 8.4). Birth dates were estimated from pouch young head lengths using the nomogram established by Lyne and Verhagen (1957). At particular visits in three (1990, 1991 and 1993) of the five years, all mature females examined had pouch young, and high proportions (0.86 in June 1989 and 0.95 in July 1992) of females with pouch young to total mature females were recorded in the other two years. Given a gestation length of approximately 18 days, breeding commenced in March of each year, following a period of several months of no breeding activity which varied but at least extended through December to February in all years except 1990, when three births were recorded in December. With the exception of July 1991, births were recorded in all months from April to November each year. The main breeding pulses occurred every autumn with secondary and less regular phases of activity in spring. Testicular tone in mature males was noticeably increased at the time of onset of female breeding activity.

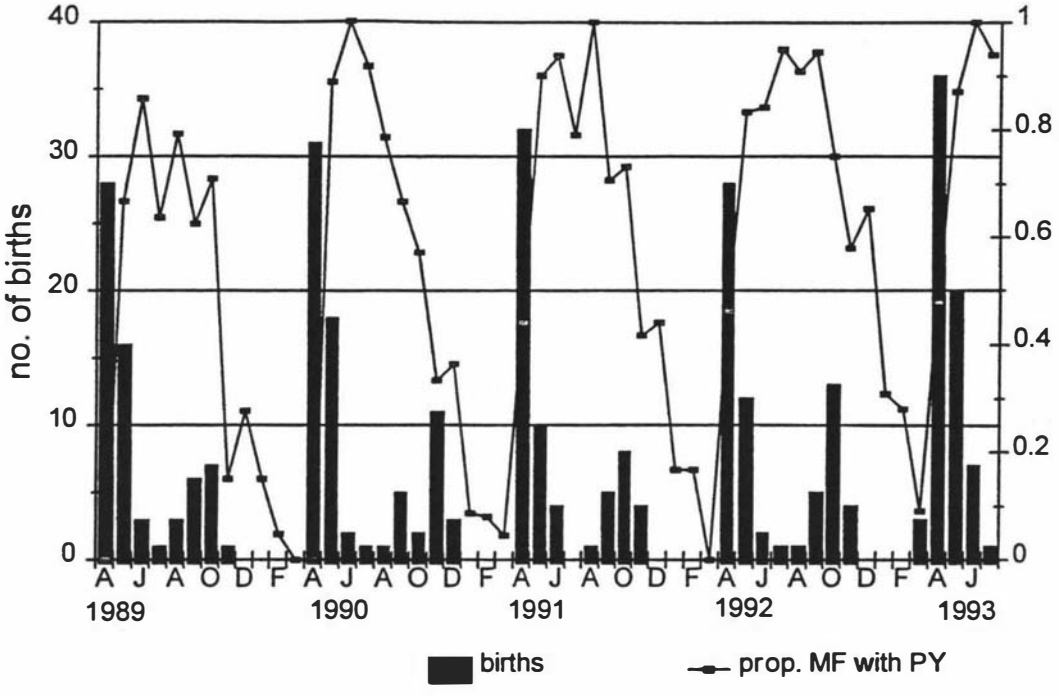


Figure 8.4. Temporal distributions of births and periods of rearing pouch young in mature female possums

Birth dates were able to be calculated for 336 pouch young. From a total of 1090 observations of mature females, separate pouch young rearing episodes were recorded on 605 occasions for 157 of 186 mature females. The distribution of the number of rearing episodes per individual are shown in Table 8.2.

Table 8.2. Number of rearing episodes per possum for 157 individual female possums over a 52 month period from April 1989 to July 1993

No. of rearing occasions	1	2	3	4	5	6	7
Individual female possums	76	37	17	12	5	8	2

Eighty-five offspring were known to have reached independence and they were offspring of 58 possums. Thirty-five of these 58 females successfully reared offspring to independence once, 20 on two occasions, and multiple rearings were successful for two females on three and for one on four occasions.

One hundred and seventy-four sequences of two rearing episodes were recorded for 81 mature females. The interval between successive births varied from 30 to 1121 days, the mean interval

was 274 days and the median was 211. The distribution of births within 30 day intervals, illustrated in Figure 8.5, show that most of the possums in this group bred at 5 to 7 month intervals rather than at 10 to 13 month intervals.

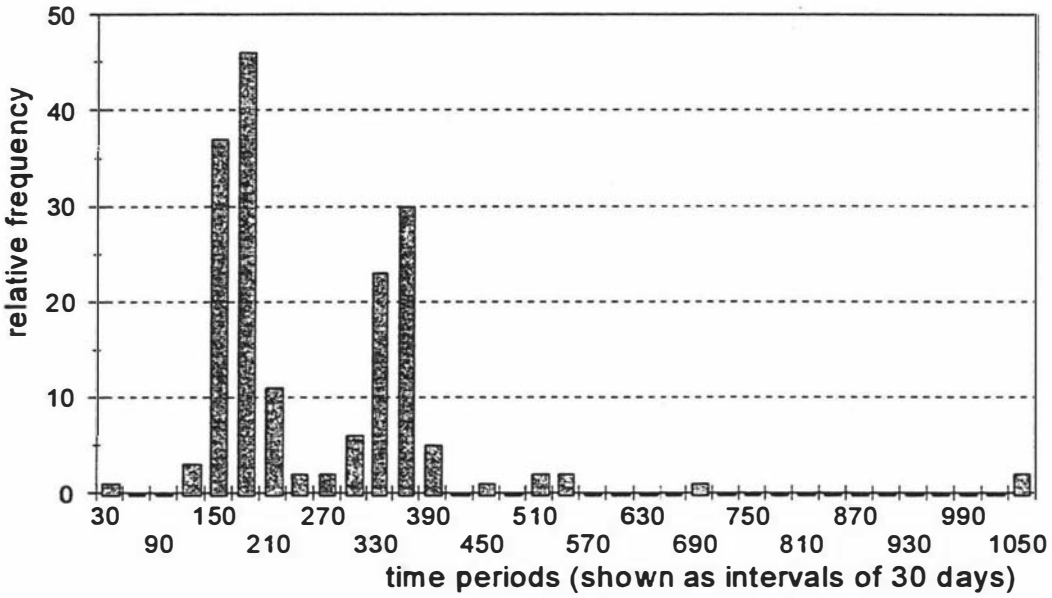


Figure 8.5. Relative frequency of periods between successive births measured in 30 day interval periods

For 63 parities where the pouch young successfully achieved independence, pouch young spent between 115 and 338 days in close relationship with their mothers (mean = 218, median = 215, s.d. = 39), based on the time of birth to time of first examination at which the mother did not have a pouch young.

Twenty-one rearing episodes were recorded for 14 of 35 female possums which were first identified as joeys and whose birthdate could be calculated from joey measurements. Using pouch young measurements, birthdates were calculated for those 21 episodes. The age at which births commenced in this cohort varied from 378 to 768 days (mean = 488, median = 409). One possum had four offspring born at 393, 694, 896 and 1070 days of age, another had three at 730, 1079 and 1484 days, and two had two at 544 and 921 days, and 508 and 704 days respectively. Males identified as joeys and with known birth dates reached sexual maturity as estimated by testicular width of at least 14 mm on average at 537 days (median = 551, min = 337, max = 762, N = 11). The average weight was 2 kg and average testicle size was 14 mm at that stage. Subsequent changes in mean body weight and mean testicle size over time are shown in Figure 8.6.

Of 80 possums which were identified as pouch young and reached independence and for which birth dates could be calculated, 23 died between 6 and 43 months (mean = 18) after birth. Of the cohort of 23 which died, five died from tuberculosis between 11 and 28 months (mean = 17.4) after birth and four died from cardiac haemorrhage subsequent to blood collection between 10 and 23 months (mean = 16.5) of birth. Of 51 born before autumn 1992, 21 died and 20 disappeared (defined as no evidence of death or not caught after visit 46) between 5 and 32 months after birth (mean = 12.5).

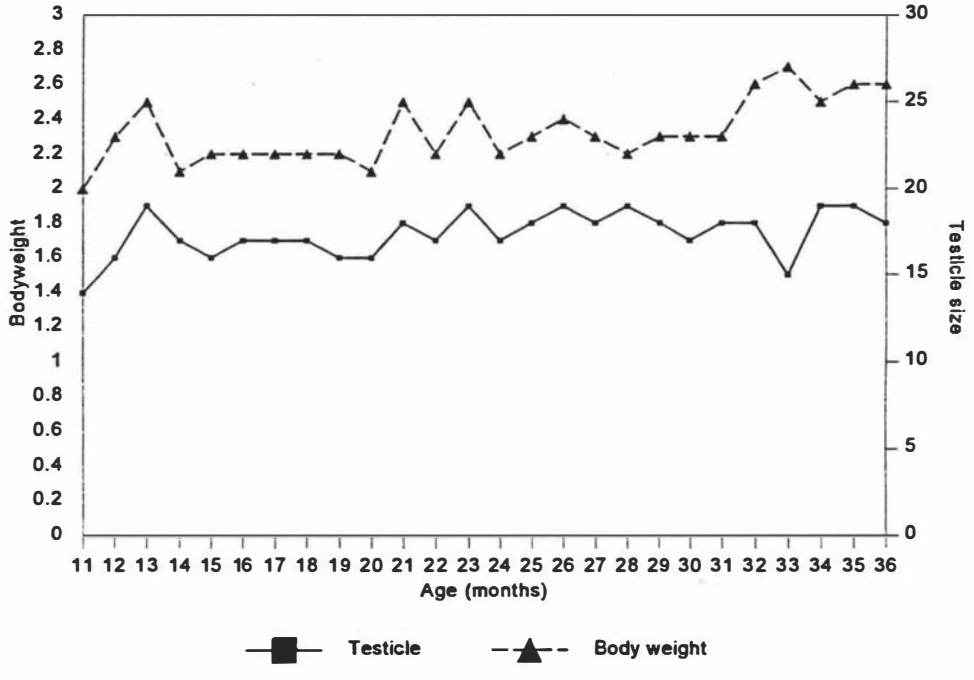


Figure 8.6. Changes in mean bodyweight and mean testicle size following independence in male possums identified as pouch young

The proportions of juveniles within total new captures per visit were regularly low during winter and high during the summer months. Four year aggregated data were used to produce the temporal distribution of the proportion of immature to mature possums in the catch of new possums each month illustrated in Figure 8.7.

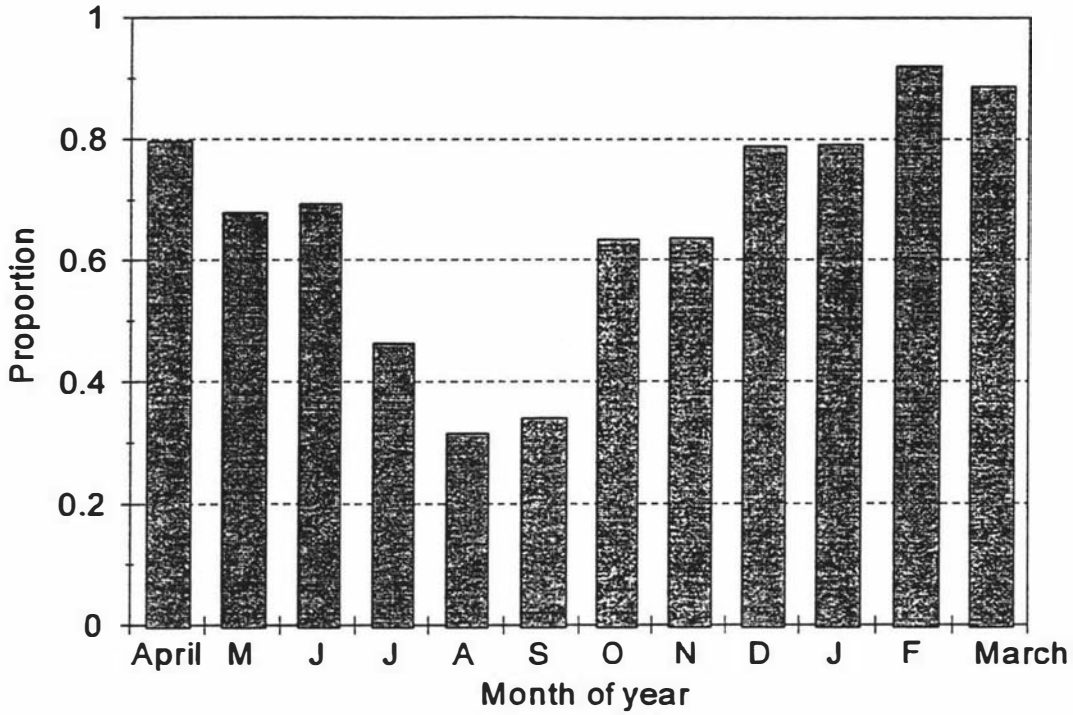


Figure 8.7. Distributions of the proportion of immature possums to mature possums in the catch of new possums each month aggregated over four years

Population dynamics

Summary information was calculated for all visits beginning at visit five, when monthly trapping effort first exceeded 1000 trap units, ending at visit 50. Summary Jolly-Seber estimates of survival probability between successive visits, population size and immigration plus births for each month are set out in Table 8.3 and the temporal changes are illustrated in Figure 8.8.

Table 8.3. Summary Jolly-Seber statistics for survival probability between successive visits, population size and immigration plus births for each month from and including visits 5 to 50.

	mean	median	s.d.	min	max
Survival probability	0.96	0.95	0.03	0.81	1.0
Population size	162	164	139	134	191
Immigration plus births	3	2.8	1.4	1.1	10.2

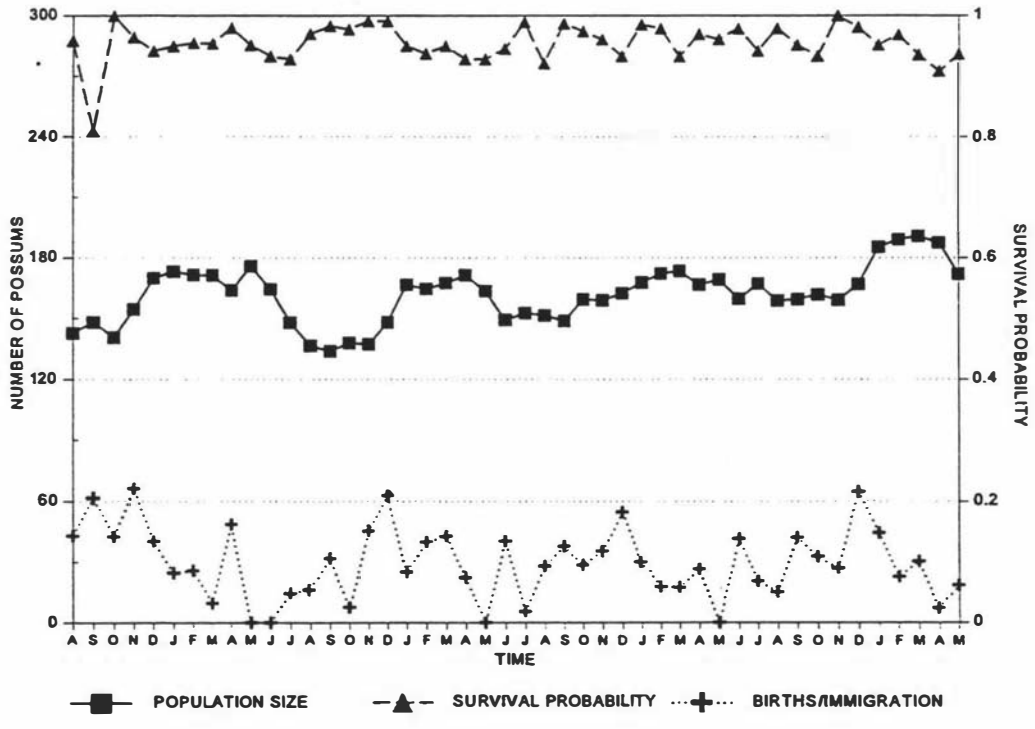


Figure 8.8. Temporal dynamics of Jolly-Seber population parameters for the possum population from visit number 5 to visit number 50

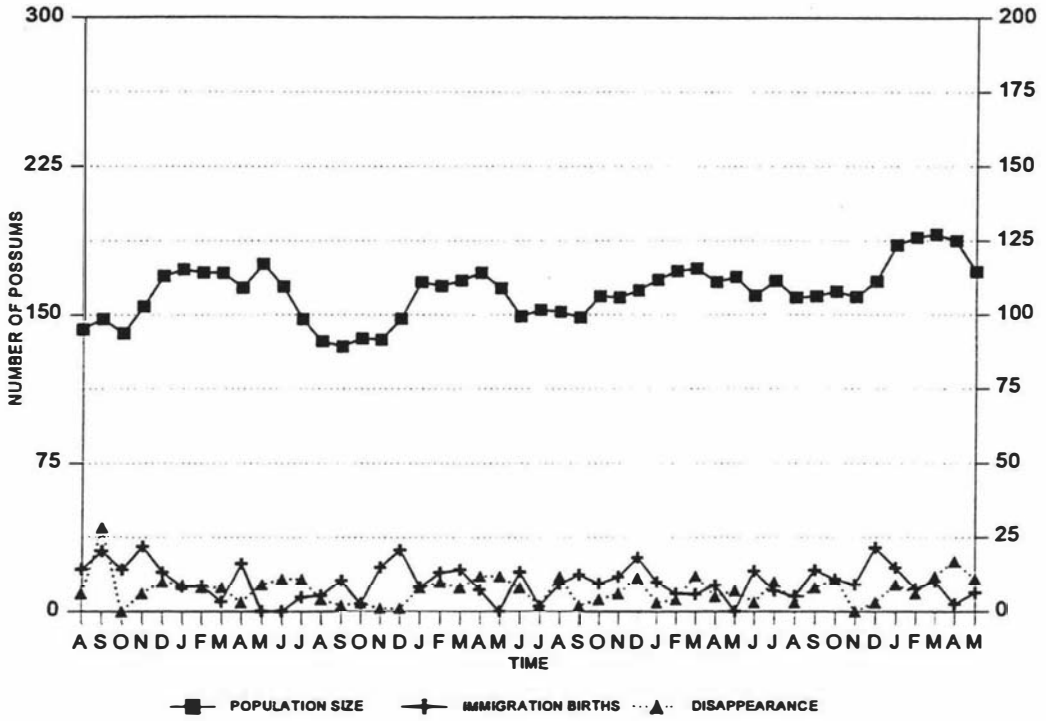


Figure 8.9. Temporal dynamics of Jolly-Seber population parameters for the possum population from visit 5 to visit 50 showing the relationship between immigration/births and disappearance

In Figure 8.9, survival probabilities were converted into numbers of animals disappearing each month and the Y axis was scaled down to give an improved presentation of the comparison of immigration/birth with disappearance. Immigration/births were greater than disappearances in 42 of the 46 months shown in Figure 8.9.

General body condition

Adult possum body weight ranged from 1.5 kg to 4.0 kg (mean = 2.5, N = 241) in males and 1.4 to 3.5 (mean = 2.4, N = 185) in females. The temporal pattern of monthly recorded bodyweights are shown in Figure 8.10 and aggregated four-year data were used to construct Figure 8.11 which emphasises the annual cyclical pattern. Mean monthly body weight of mature male possums was positively correlated with Jolly-Seber population size estimates (r = +0.55) but showed no correlation with survival probabilities (No of visits = 46).

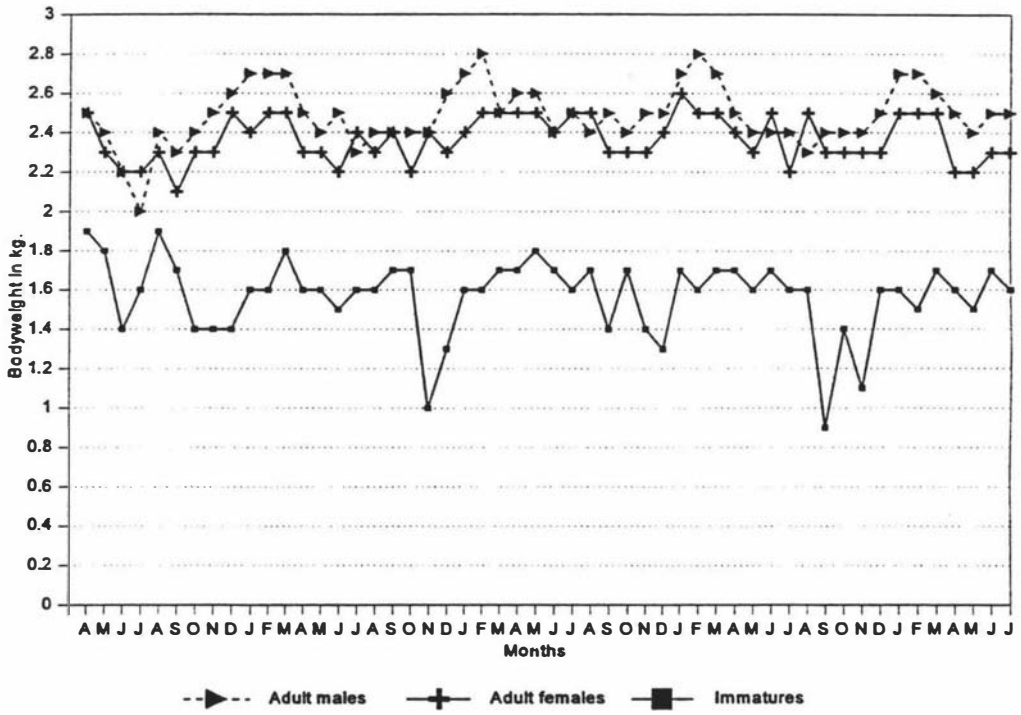


Figure 8.10. Temporal pattern of average body weights of mature male and female and immature possums over 52 months.

Monthly bodyweight data, aggregated over a four year period was used in Figure 8.11 which was designed to emphasize the regular temporal changes in bodyweight of mature male and female possums. Repeated measures analysis of variance was used to analyse the effects of season (Spring was designated = September, October and November) and sex on bodyweight. For the analysis, possum identity was nested within the fixed variables, sex and season. The results indicated that sex (F-ratio = 24.12, $p < 0.001$), season (F-ratio = 122.52, $p < 0.001$) and interaction between sex and season (F-ratio = 14.42, $p < 0.001$) significantly influenced bodyweight. A total of 2557 observations from 425 possums were used in the analysis.

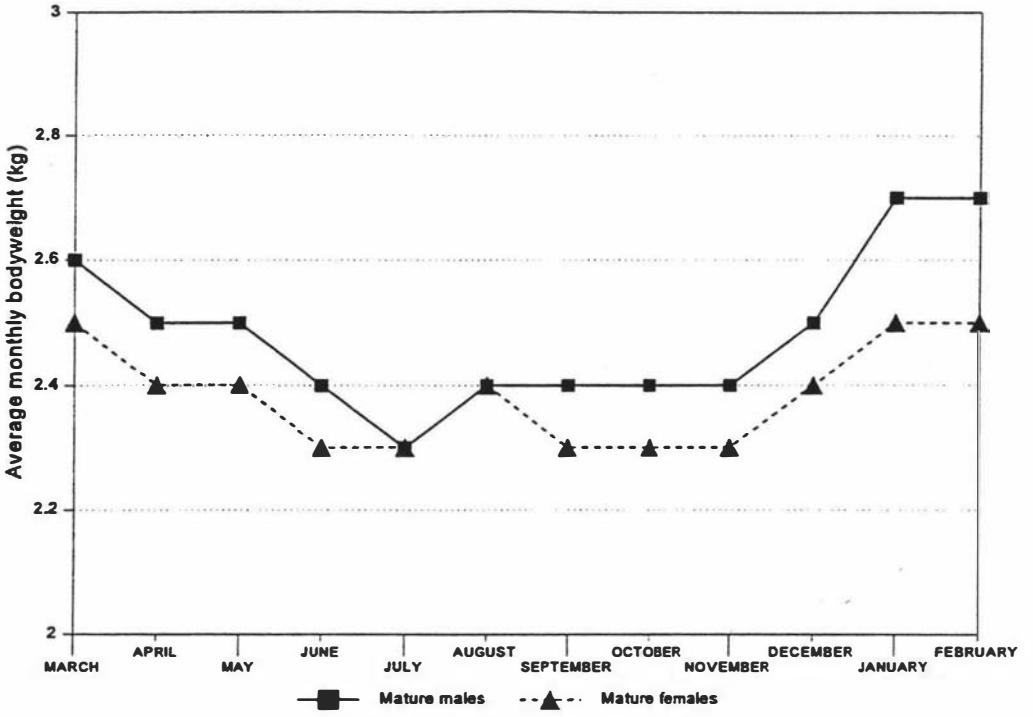


Figure 8.11. Temporal patterns of bodyweights of adult male and female possums (note restricted range of values shown on the Y axis)

Denning

During the period from 19/07/89 to 31/07/93, radio-collars were attached to 147 possums, which enabled tracking to a total of 595 den sites on 818 occasions. Collars were attached to all possums with clinical signs of tuberculosis and purposive selection also predominated for the remainder, based on needs to obtain information about behaviour of dispersing juveniles and to determine extent of home ranges. On occasions when logistics permitted, radio-collars were fitted to randomly selected possums.

Individual possums were tracked from one to 44 times (mean = 5.6) and used from one to 32 separate dens (mean = 4.6). There was a strong correlation (r = +0.98) between trapping effort and the number of separate dens recorded per possum (Figure 8.12.).

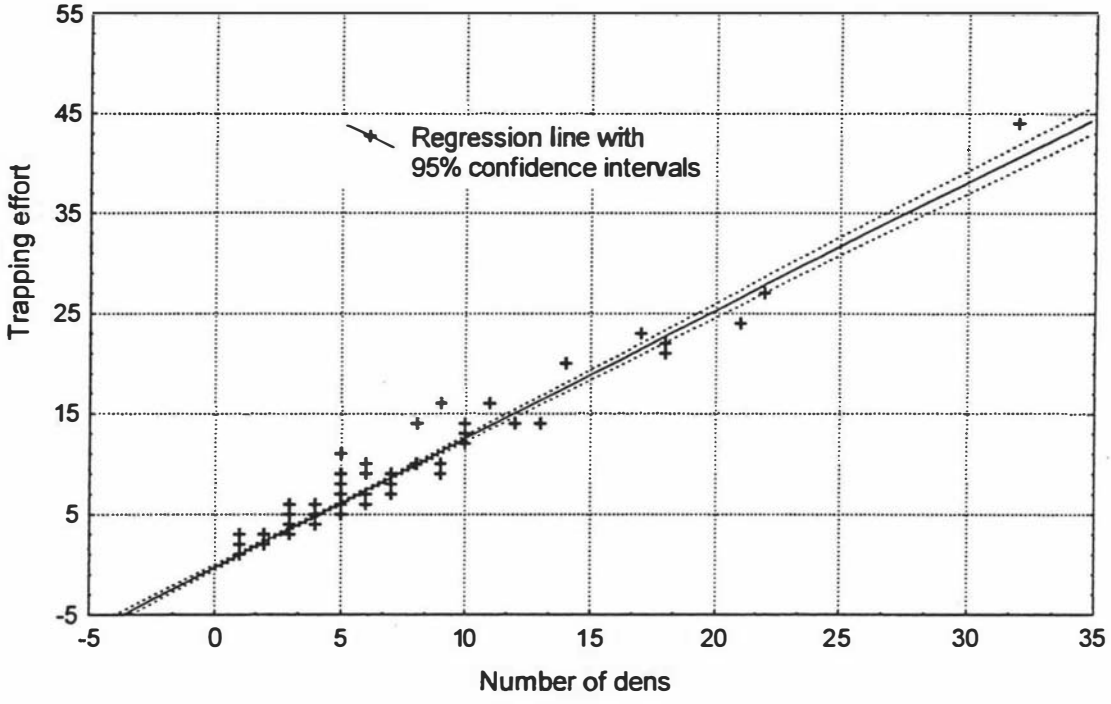


Figure 8.12. Scatterplot of den-site tracking effort (N = 818 occasions) and the number of different dens (N = 595 den-sites) used by individual possums

Between 20/01/91 and 31/07/93, the type of den used, and the weather on the day of tracking, and on the previous day was recorded. In that 30 month period, 67 possums were tracked on 358 occasions to 281 separate dens and individual animals were tracked from one to 23 times (mean = 5.2).

Of those 281 dens, 133 (47%) were located in flax plants (*Phormium tenax*), 45 (16%) in bulldozed root-rakings formed during scrub clearing, 39 (14%) in gorse (*Ulex europaeus*), 24 (9%) in grass shelters, eight (3%) in underground burrows, six (2%) in ponga tree ferns (*Cyathea dealbata*), ten (3%) in pine trees (*Pinus radiata*) one in a cabbage tree (*Cordyline australis*) and the remaining 15 (5%) in miscellaneous locations.

One root-raking den was occupied sequentially by three different possums and 13 other dens (four flax, five root-raking, one burrow, one pine tree and 2 gorse dens), were used sequentially by different individuals twice. Many of the root-rakings were large (up to six metres by three metres) and contained extensive connecting tunnel-like spaces.

No associations were found between the type of den occupied and weather, either on the day of tracking or on the previous day.

Immigration and a comparison of known locally recruited possums and immigrants

During the 36 month period from January 1991 to December 1993 inclusive, on average 6.4 juvenile possums (comprised of those pouch young previously identified plus others whose origins were unknown) and 2.1 new adults were captured each visit. Over 52 months of the study, the proportion of males in new juveniles was 0.73 (225 of 309) and the proportion of males in juveniles known to be locally recruited was 0.54 (47 of 87), a statistically significant difference ($p = 0.001$, OR = 0.44, 95% c.i. 0.26 - 0.74); see Figure 8.13. Figure 8.14 shows no clear pattern for capture histories subsequent to the first capture for all possums.

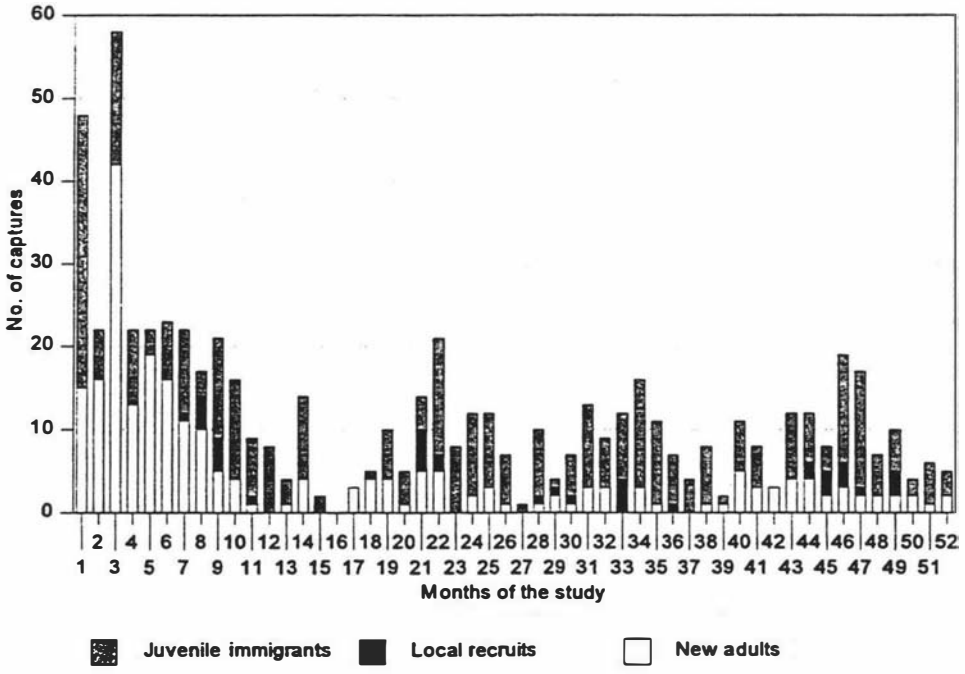


Figure 8.13. Captures of new possums stratified by age groups

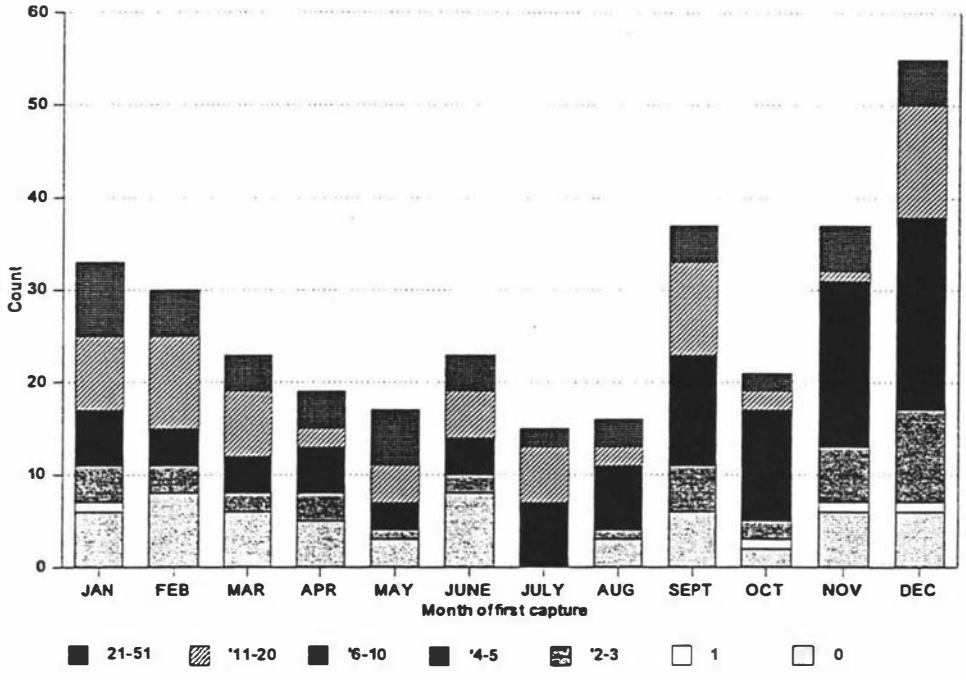


Figure 8.14. Aggregated 36 month data showing the number of months for which possums were captured after initial capture depending on month of capture

Dispersal

Five immature males, one mature male and one immature female possum were tracked and recovered at new locations up to 2.2 km from the main denning area in the study site and one male was trapped in a gully 1200 metres away (summary statistics for the dispersal distances were mean = 1120, median = 1090, max = 2200, min = 250 metres). Four other possums (3 immature males and one immature female) were tracked to locations off the study site (in one case, 1000 metres distant) before being lost, either due to radio-collar failure and/or dispersal into regions of difficult terrain which could not effectively be checked for signals. Fourteen other possums (5 immature males, 6 mature males, one mature and 2 immature females) which had radio-collars fitted were lost to follow-up during the 52 months of the study.

TUBERCULOSIS EPIDEMIOLOGY

Descriptive epidemiology

During the 52 months of the study under consideration, 59 of 632 possums were diagnosed tuberculous by cultural examination for *M. bovis*. The period prevalence for those 52 months was 0.093 (95% c.i. 0.072 - 0.119). Average monthly prevalence was 0.06 and ranged from 0 to 0.20. Zero prevalences were recorded in April 1989 and in August and October in 1992. Average monthly cumulative incidence was 0.02 and ranged from 0 to 0.17. Monthly incidence density was 0.008. Of the tuberculous possums, 28 males and 20 females were mature and seven males and four females were immature at the time of initial diagnosis of disease. The proportion of animals which were found to be tuberculous was 0.20 in mature and 0.03 in immature possums (OR = 8.91, 95% c.i. = 4.35 - 18.65, $p < 0.001$) based on the time that the initial diagnosis was made (see Figure 8.15.). There was no difference in the risk of becoming infected between males and females (OR = 1.11, 95% c.i. = 0.62 - 1.97,). Within sex comparisons stratified on age showed that the risk was higher for mature animals in both males (OR = 7.24, 95% c.i. = 2.87 - 19.03, $p <0.001$) and females (OR = 9.75, 95% c.i. = 3.11 - 33.98, $p <0.001$). In the early part of the study 5 of 7 dead possums which were found on pasture and which had not been previously tagged were shown to be tuberculous following cultural examination.

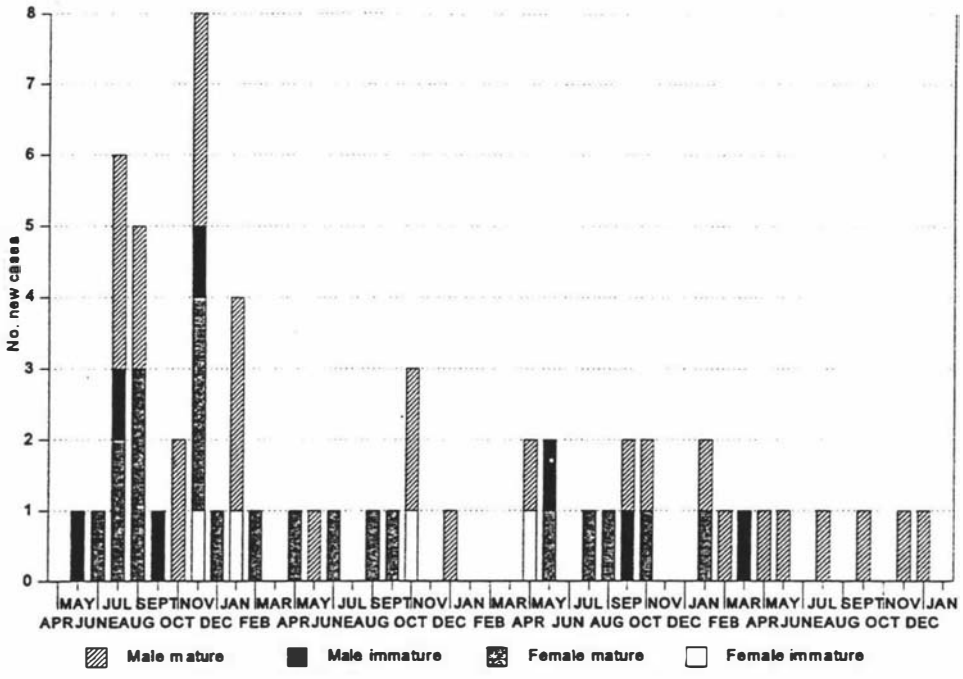


Figure 8.15. New cases of tuberculosis over the first 45 months of the study, stratified by age and sex (no new cases were recorded between visits 46 and 52)

There was no significant difference between the mean ages of tuberculous possums which died and for which age estimations could be made (N = 49, mean = 24.0 months, median = 19, s.d. = 17.61) and those which died from other causes (N = 131, mean = 23.3 months, median = 15, s.d. = 21.3). There were no statistically significant differences between the age at death of tuberculosis affected and non-affected males (mean Tb +ve = 19.5, Tb -ve = 18.5, p = 0.37) and tuberculosis affected and non-affected females (mean Tb +ve = 29.0, Tb -ve = 29.7, p = 0.46). A significant difference (p = 0.04) was found between the ages at death for tuberculosis affected males (mean = 20 months, N = 25) and tuberculosis affected females (mean = 29, N = 24).

No differences were found between the temporal incidences in mature females with and without pouch young, (Figures 8.16a. and 8.16b.).

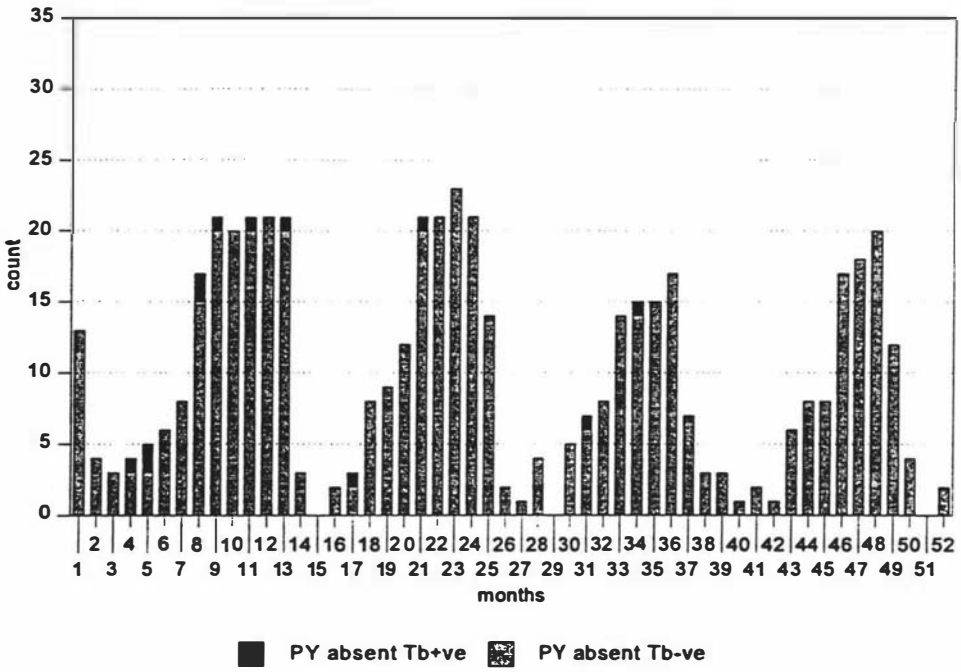


Figure 8.16a. Temporal distribution of incident cases of tuberculosis in mature females without pouch young present

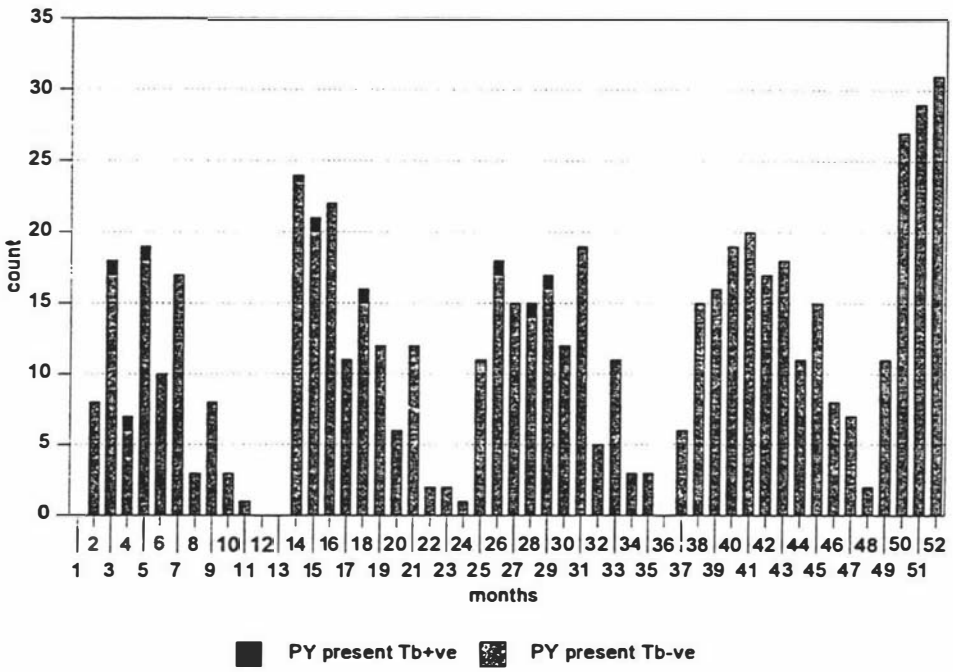


Figure 8.16b. Temporal distribution of incident cases of tuberculosis in mature females with pouch young present

During the 52 month period, 16 female possum which were found to be infected with tuberculosis had rearing episodes recorded. Seven of these females reared ten offspring which were subsequently trapped as independent animals. The summary histories of those seven possums and one other, D3728, whose disease status was never determined are set out in summary form below: The abbreviations BL, CP, and LTT indicate Blocking assay, Culture filtrate assay, and Lymphocyte transformation test.

D3513, mature when first trapped on 16/06/89

Mother D3513 Rea Type 4a	1989	**Female joey (D3694)**,first trapped as independent on 12/12/89 at 8 months, LTT+ on 11/12/90 and 23/4/91, died misadventure 22/04/91, generalised Tb, aged 24 months without breeding, Rea Type 4a
Diagnosed clinical Tb 09/04/90 BL and CP + 23/10/90 Died Tb 18/01/91	1990 1991	**Female joey (D2910)**, independent 12/12/90, last trapped 25/02/91 at age 10 months - then lost Tb status unknown

D3709, mature when first trapped on 20/08/89

Mother D3709 Rea Type 4b	1989	**Female joey (D3684)**, first trapped as independent 12/12/89, LTT + on 16/01/90, died misadventure aged 11 months on 15/03/90, grossly negative at necropsy - no cultural examination done
BL + 30/07/91	1990	Had a female pouch young which was not identified
Died Tb 23/10/91	1991	Had a female pouch young which was not identified

D3712, female, mature when first trapped on 20/08/89

Mother D3712 Rea Type 4a	1989	**male joey (D2270)**, first trapped as independent on 17/11/89, still alive aged 52 months with no signs of Tb at end of study period
	1990	**female joey (D2906)**, first trapped as independent on 10/12/90, diagnosed Tb 22/04/91 and died on 08/05/91, aged 13 months, Rea Type 4a
	1991	joey died with mother and was not suitable for cultural examination

Diagnosed clinical Tb 07/08/91
Died Tb 04/10/91

D3719, mature when first trapped on 26/09/89

Mother D3719	1989	No pouch young
Rea Type 4a	1990	Pouch young in both autumn and spring but not identified
BL and CP + 12/11/91 LTT + 21/01/92	1991	**male joey (D2992)** first trapped as independent on 23/10/91, LTT + on 13/3/92 at 11 months, died Tb 07/05/92, aged 13 months, Rea Type 4a
Diagnosed clinical Tb 21/01/92	1992	
Died Tb 25/03/92		

D3720, mature when first trapped on 26/09/89

Rea Type 4	1989	**female joey (D3692)**,first trapped as independent on 17/11/89, died Tb , Rea Type 4 10/01/90, aged 9 months

Diagnosed clinical Tb 16/11/89
Died Tb 10/01/90

D2471, mature when first trapped on 22/05/89

Mother D2471	1989	**male joey (D3578)**,first captured as independent on 27/10/89 and then lost

Died Tb 10/12/89 with no history of clinical Tb

D3728, female, mature when first trapped on 29/09/89

Mother D3728	1989	**female joey (D3644)** first captured as independent on 29/08/90, aged 16 months and was diagnosed Tb at that time, LTT + on 11/12/90 and 31/07/91, died Tb 10/09/91, aged 17 months

D3728 was last trapped on 09/04/90 and was not seen again. Clinical Tb was not diagnosed in this animal

D3680, mature when first trapped on 14/11/89

Mother D3680, Rea Type 4a	1990	autumn born female joey, not identified
BL + on 12/03/90, 27/05/90		spring born male joey, not found again
and 23/07/90	1991	autumn born joey, necropsied when approximately 70 days
LTT + on 28/05/91		old. *M. bovis* was isolated from a sample of liver and
Diagnosed clinical Tb 27/05/91		lung, Rea Type 4a
Died Tb 29/07/91		

In all cases where lesions from both mother and offspring were cultured (mothers D3513, D3712, D3719, D3720 and D3680), the restriction endonuclease types were identical.

Pathology observations

Physical evidence of death was available for 53 of 59 possums from which *M.bovis* was isolated. Five of those tuberculous possums were found dead on pasture and had no previous capture history. One tuberculous possum which was first diagnosed tuberculous on visit month 45 was still alive at the end of the 52 month period. Five tuberculous possums were lost to follow up when radio-collar transmissions could no longer be detected.

During routine monthly clinical examinations, palpable lesions were found in 43 (78%) of 55 tuberculous possums. Discharging fistulas were recorded in 22 (37 %) of 59 tuberculous possums and in four of those animals (18 %), the fistula openings were subsequently found to have healed. Most of the possums available for necropsy died naturally from tuberculosis at a terminal stage of the disease process. They were characterised by generalised and advanced disease with lesions present in most organs. Lung lesions were extensive and disseminated and commonly occupied about one third of the volume of lung tissue. Further more detailed descriptions of the pathology and pathogenesis of the disease are presented in a separate chapter (Chapter 5) dealing with pathogenesis.

Survival of possums

Fifty nine possums died due to cardiac haemorrhage during routine blood collections which were performed on a total of 3247 occasions. Unweighted logistic regression conducted in Statistix, Version 4.0 was used to test for associations between the number of times individuals were bled (mean = 5.3, min= 1, max = 25), sex, and the aftermath of blood collection. A lower risk of a fatal outcome was shown for males (O.R. = 0.5, 95% c.i. = 0.29 and 0.88, p = 0.01) but no association was found between the number of times individual possums were bled and outcome. When the proportions of fatal collections were compared, the relative risk for female collections

was 0.54 (95% c.i. = 0.43 and 0.66, p = < 0.001). The Kaplan-Meier survivor function curves for possums which died from misadventure (N =59) and others (N =328) excluding those lost to follow-up (N = 243) are shown in Figure 8.17. The log-rank test showed a significant difference between the groups ($\chi_1^2 = 51.41$, p = <0.001). Because of the potential confounding effect from 59 possums which died from misadventure, they were excluded from other survival analyses. In those analyses, survival function estimates were based on the time of confirmed death, the time of loss to follow-up and the last time they were trapped. In the absence of evidence of death, possums were ruled to be censored if they had been trapped in the last 6 months of the study and ruled to be lost to follow-up if they had not been seen after the 46th month. This compromise was chosen because the figure for known deaths was considered to be an underestimate and the one for loss to follow up an overestimate. Possums were credited with one month's survival at their first trapping occasion, meaning that animals which were captured on one occasion only were considered to have survived for that month.

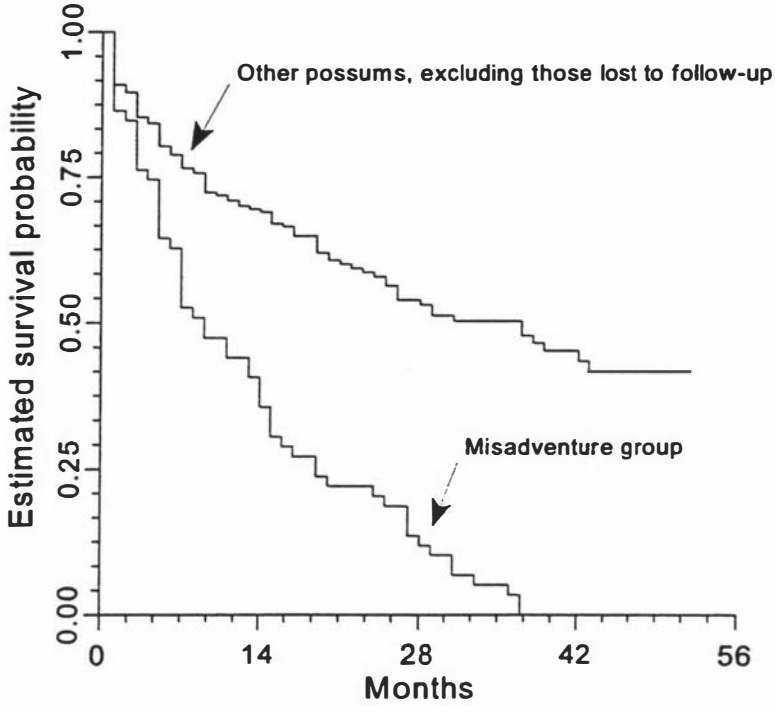


Figure 8.17. Estimated survivor functions for possums which died from misadventure and possums which were not lost to follow-up

Survivor function curves (Figure 8.18) were constructed for non -tuberculous possums not lost to follow-up (N =76 failed, 205 censored), possums lost to follow-up (N =243) and tuberculous possums (N =43 failed, one censored). An overall log-rank test showed significant between group differences (χ_2^2 = 176.61, p = <0.001), and a stratified log-rank test showed no significant differences (χ_1^2 = 1.2, p = 0.3) between the lost to follow-up group and the tuberculous group.

Table 8.4 sets out the survivor functions for possums in those groups at six month intervals and the end of the study period. The table shows that a possum in the non-tuberculous group had an 0.65 (95% c.i. 0.59 - 0.71) probability of survival to 30 months or longer, while the survival probability of tuberculous animals was zero at that time.

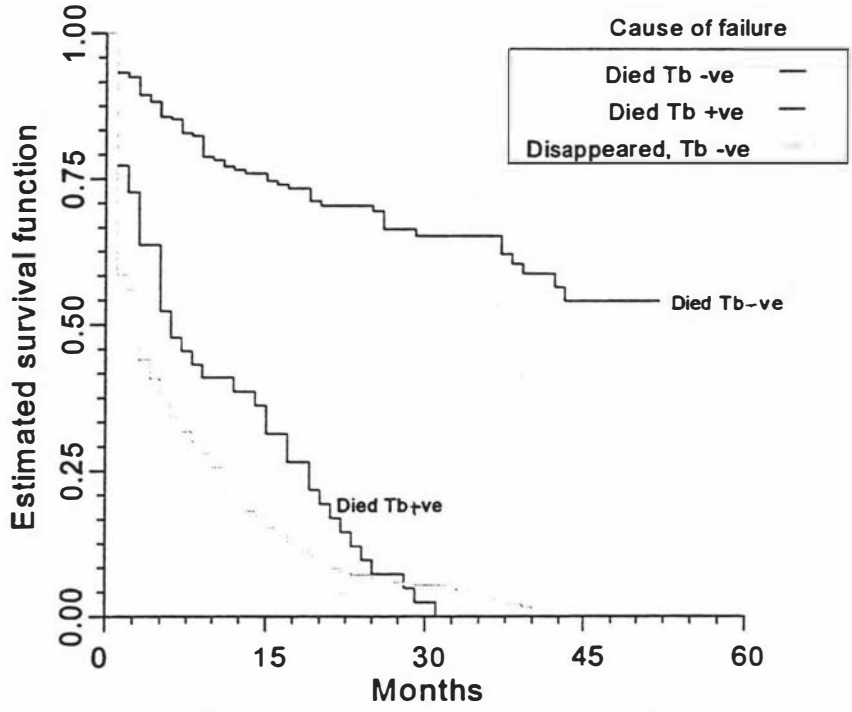


Figure 8.18. Kaplan-Meier survivor functions for infected and non-infected possums

Table 8.4. Survival functions *S (t)* for non-tuberculous possums which remained in the study (N = 76 failed, 205 censored), non-tuberculous possums which disappeared (N = 243 failed) and tuberculous possums (N = 43 failed, one censored)

Month	Tb -ve and not lost		Disappeared Tb -ve		Tuberculous possums	
of study *(t)*	*S (t)*	95% c.i.	*S (t)*	95% c.i.	*S (t)*	95% c.i.
1	1.00	0.99 - 100	1.00	0.99 - 1.00	0.98	0.93 - 1.00
6	0.85	0.81 - 0.89	0.36	0.30 - 0.42	0.50	0.35 - 0.65
12	0.77	0.71 - 0.82	0.21	0.16 - 0.26	0.39	0.24 - 0.53
18	0.73	0.67 - 0.79	0.12	0.08 - 0.16	0.24	0.11 - 0.37
24	0.69	0.63 - 0.76	0.07	0.03 - 0.10	0.10	0.01 - 0.19
30	0.65	0.59 - 0.71	0.05	0.02 - 0.08	0	0
36	0.64	0.56 - 0.71	0.03	0.01 - 0.05		
39	0.59	0.51 - 0.67	0.01	0.00 - 0.02		
40	0.59	0.51 - 0.67				
42	0.54	0.44 - 0.66				
48	0.54	0.44 - 0.64				
52	0.54	0.44 - 0.64				

S (t) = survivor function = the probability that an individual possum survives for a time greater than or equal to time *(t)*

Survival curves for tuberculous males (N = 23 complete, one censored), tuberculous females (N = 20 censored) and non-tuberculous males (N = 49 complete, 119 censored) and non-tuberculous females (N = 30 complete and 86 censored) were calculated and are shown in Figure 8.19.

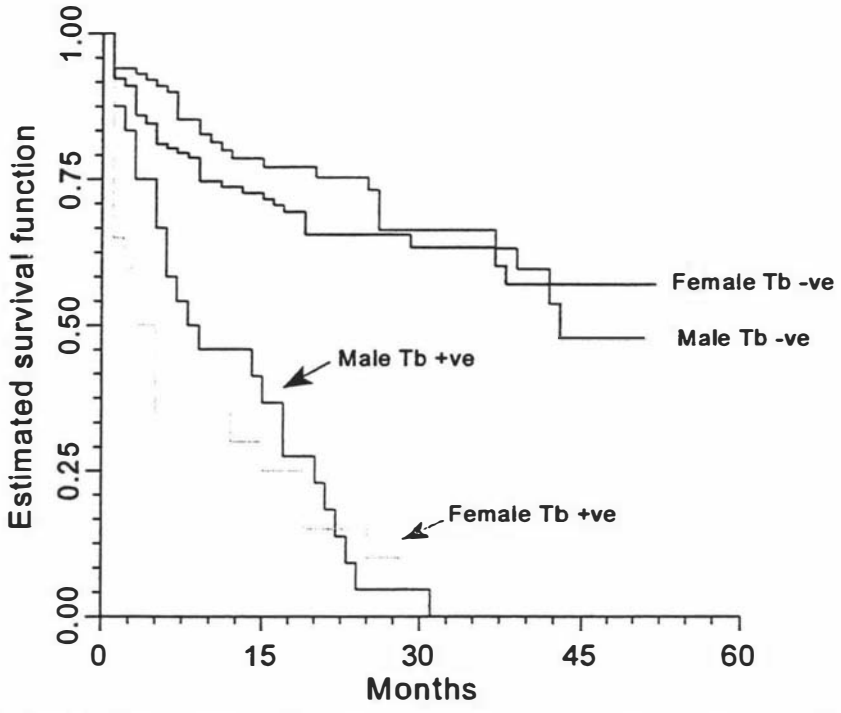


Figure 8.19. Estimated survivor function curves of groups of possums stratified by sex and tuberculosis infection status

The overall log-rank test for differences between groups was statistically significant ($\chi_3^2 = 68.59$, $p = <0.001$) but stratified log-rank tests showed no statistically significant differences between either infected males and females ($\chi_1^2 = 0.61$, $p = 0.44$), or non- infected males and females ($\chi_1^2 = 0.74$, $p = 0.39$).

Temporal dynamics of tuberculosis infection

Monthly point prevalence and cumulative monthly incidence for 52 months of study are shown in Figure 8.20. Tuberculous possums were known to be present in the study site for the whole period except at the initial visit in April 1989, and again in August 1992 and October 1992. The epidemic curves show a gradual decline in prevalence and incidence over time with peaks in point prevalence in July 1989 with 0.2 (N = 30), November 1989 to January 1990 with 0.161, 0.132 and 0.149 (N = 62, 76 and 74) respectively, November and December 1990 with 0.123 and 0.152 (N = 46 and 80), May 1991 with 0.109 (N = 64), September 1991 with 0.113 (N = 53), March 1992 with 0.067 (N = 75) and December 1992 with 0.027 (N = 73). The denominator (N) used for calculation of prevalence and incidence statistics was the number of animals clinically examined at each particular visit.

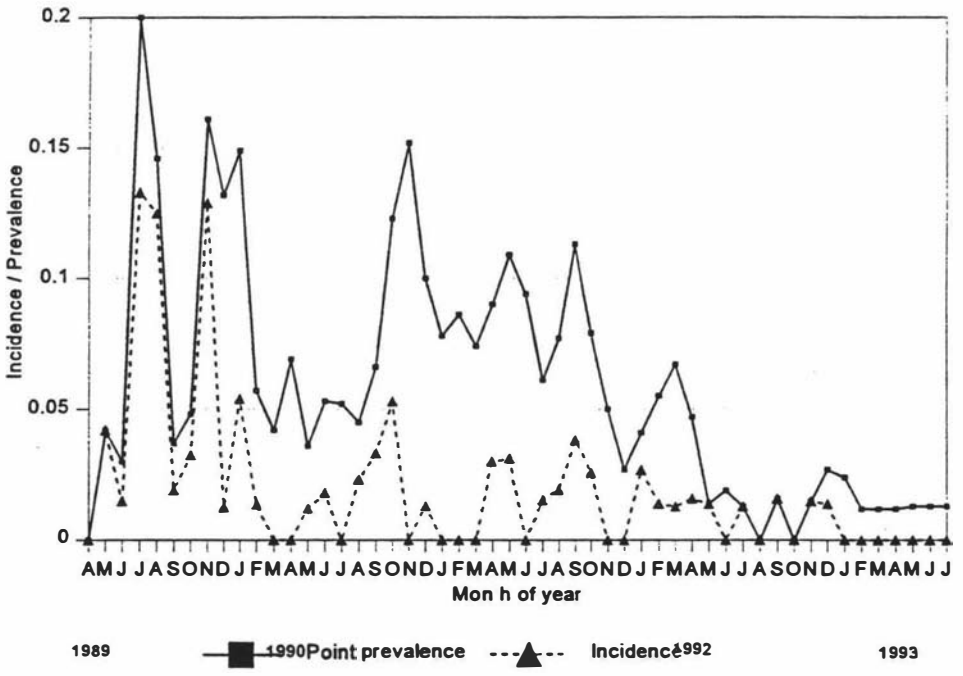


Figure 8.20. Incidence and prevalence of tuberculosis in possums at Castlepoint from April 1989 to July 1993

The average monthly prevalence was 0.06 and varied from zero to 0.20. Figure 8.21a shows average monthly point prevalences calculated from aggregated monthly data from the 52 month study period. The lowest prevalences were recorded in the period from February to June and the highest prevalence was in November. Prevalence was stratified by sex in Figure 8.21b and this figure shows that the shape of the annual pattern for males closely resembles the annual pattern for females lagged three months.

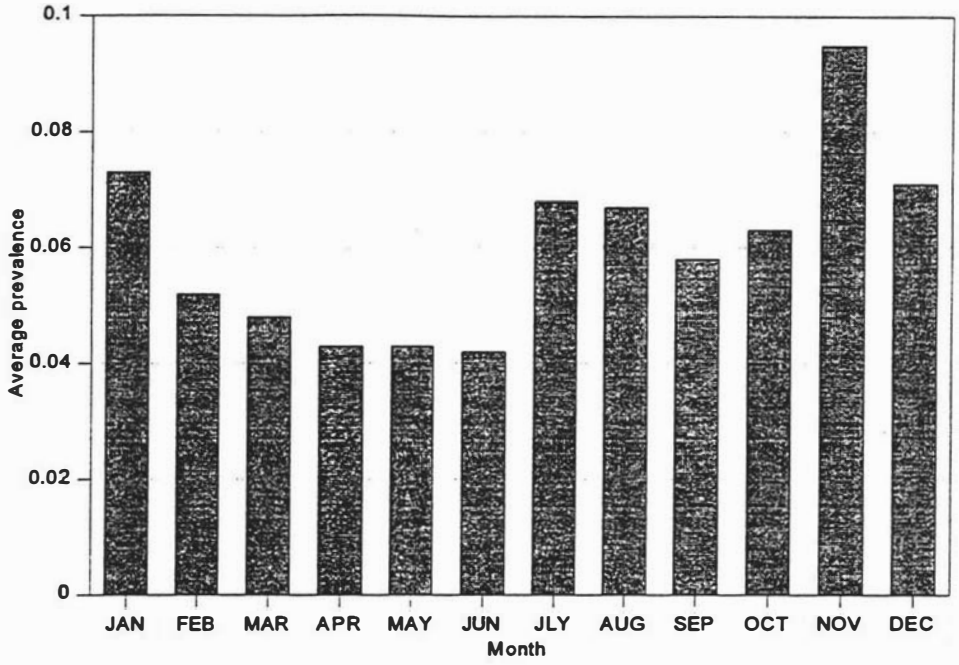


Figure 8.21a. Average monthly point prevalences calculated from data from all 52 months of the study period under consideration

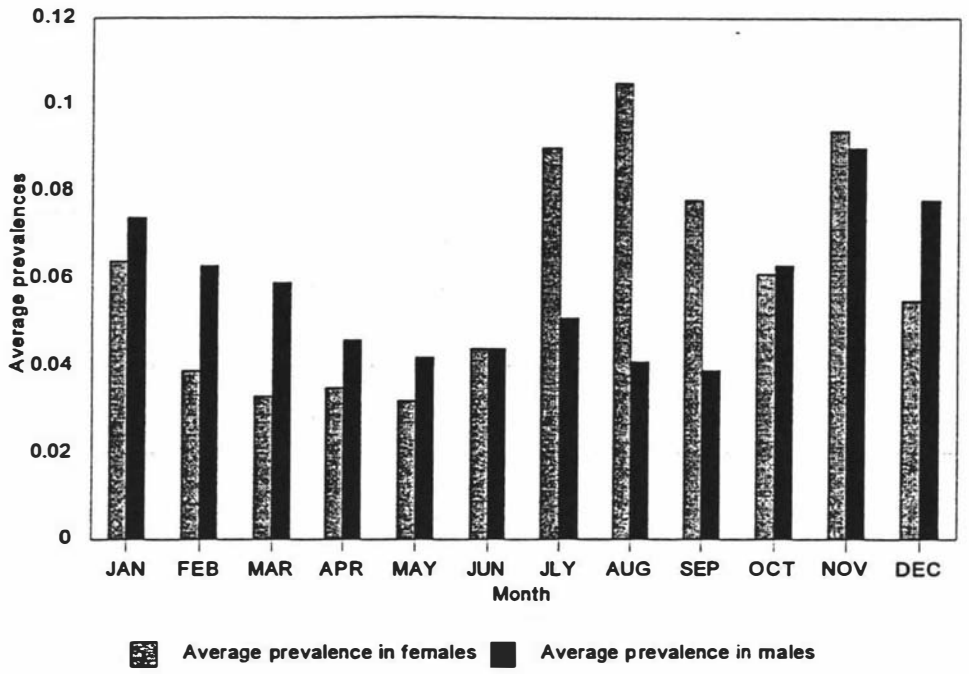


Figure 8.21b. Average monthly point prevalences for males and females calculated from data for all 52 months of the study, using the numbers of male and female possums clinically examined at each visit for calculation of the denominators

Cumulative monthly incidence showed peaks in July 1989 with 0.133 (N = 30), August 1989 with 0.125 (N = 48, November 1989 with 0.113 (N = 62), January 1990 with 0.054 (N = 74), June 1990 with 0.018 (N = 57), October 1990 with 0.053 (N = 57), April 1991 with 0.030 (N = 67), May 1991 with 0.031 (N = 64), September 1991 with 0.038 (N = 53) and January 1992 with 0.027 (N = 74). Thereafter, single incident cases were recorded in April May, July, November and December of 1992.

Aggregated monthly incidence data indicates that most new cases were found in the period between July and November each year (Figure 8.22a). Figure 8.22b shows that the pattern for the aggregated incidences in males more closely follows the overall pattern for both sexes combined than does the incident pattern for females which shows pronounced peaks in July and August. The denominators used to calculate monthly incidences stratified by sex were derived from the numbers of males and females clinically examined at each monthly visit.

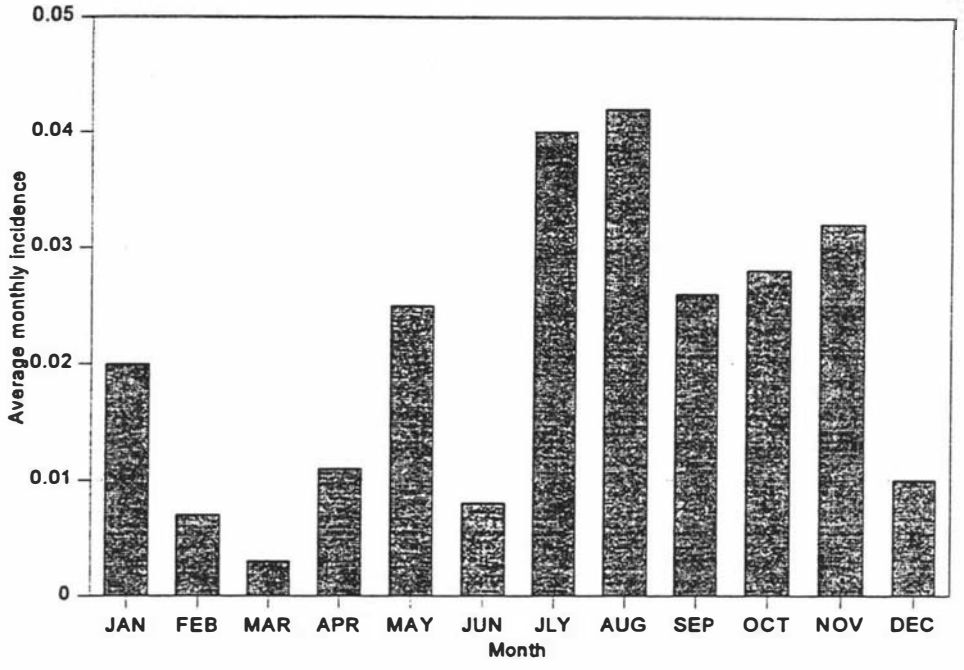


Figure 8.22a. Average monthly cumulative incidences calculated from the 52 months of the period under consideration

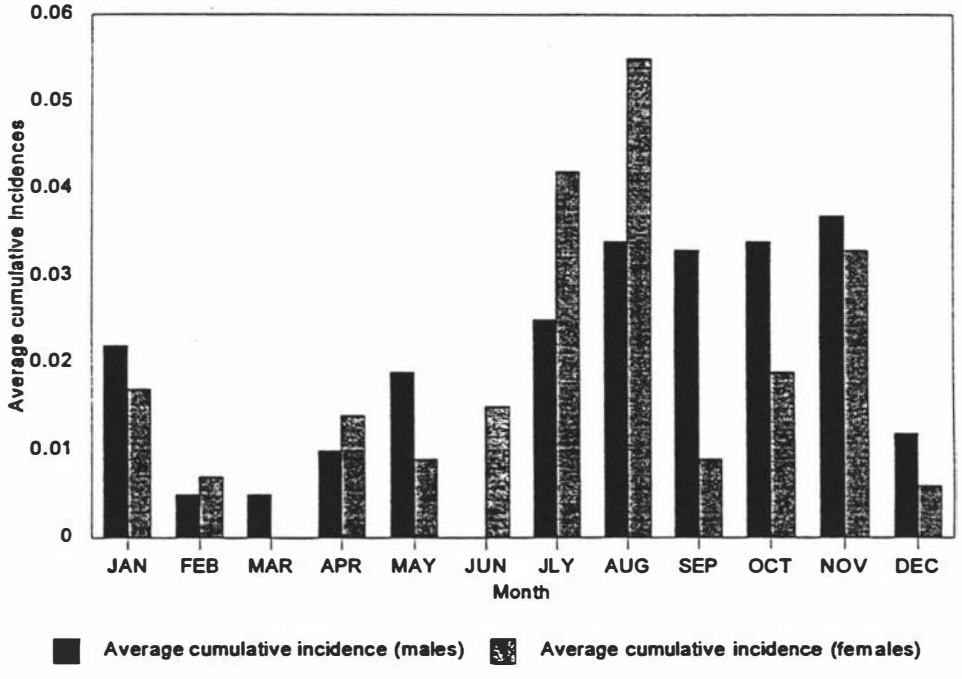


Figure 8.22b. Average monthly cumulative incidences for males and females calculated from data for all 52 months of the study period

No clear patterns were seen when the numbers of incident cases of tuberculosis were presented for mature males and mature females (Figure 8.22c) and all males and all females (Figure 8.22d), although there are indications of a trend for cases in females to occur in bursts of about three months duration prior to bursts of cases in males in the ensuing few months.

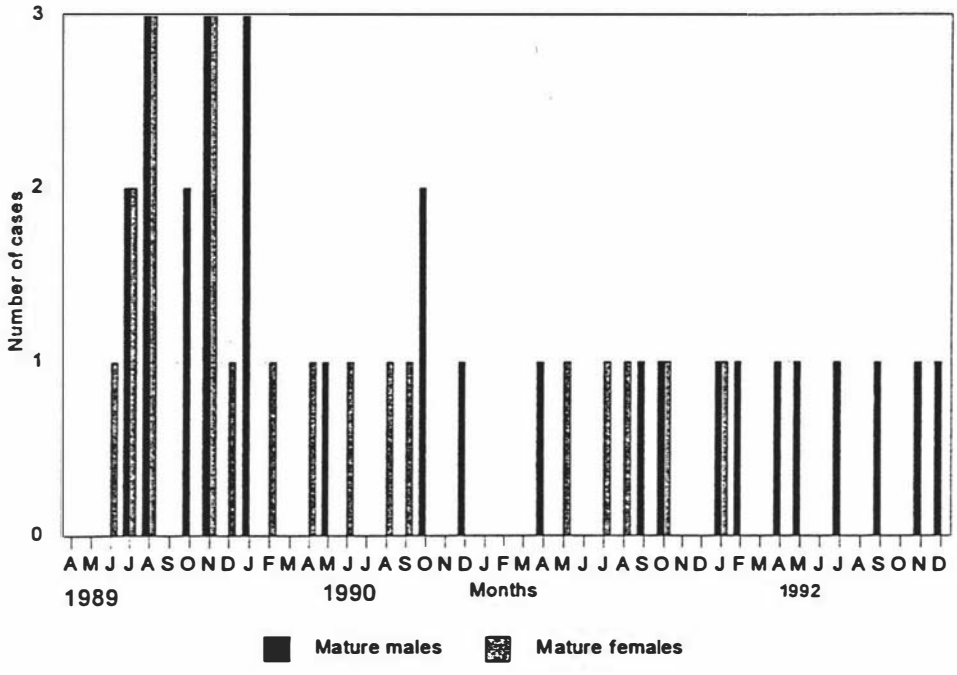


Figure 8.22c. Number of mature male and mature female incident cases of tuberculosis at each month

Figure 8.22d. Number of total male and total female incident cases of tuberculosis at each month

Epidemiological analysis based on restriction endonuclease patterns of *Mycobacterium bovis*

Mycobacterium bovis isolates from 52 possums were classified into two major and three minor patterns using restriction endonuclease analysis (Rea). Thirty isolates were classified as type 4a (similar to Wairarapa type 4), 13 as type 4 (very similar to Wairarapa type 4), four as type 10 (very similar to Wairarapa type 10), four as type 4b (similar to Wairarapa type 4) and one as type 4c (similar but not identical to type 4b). The temporal distribution of incident cases classified by Rea type is shown in Figure 8.23.

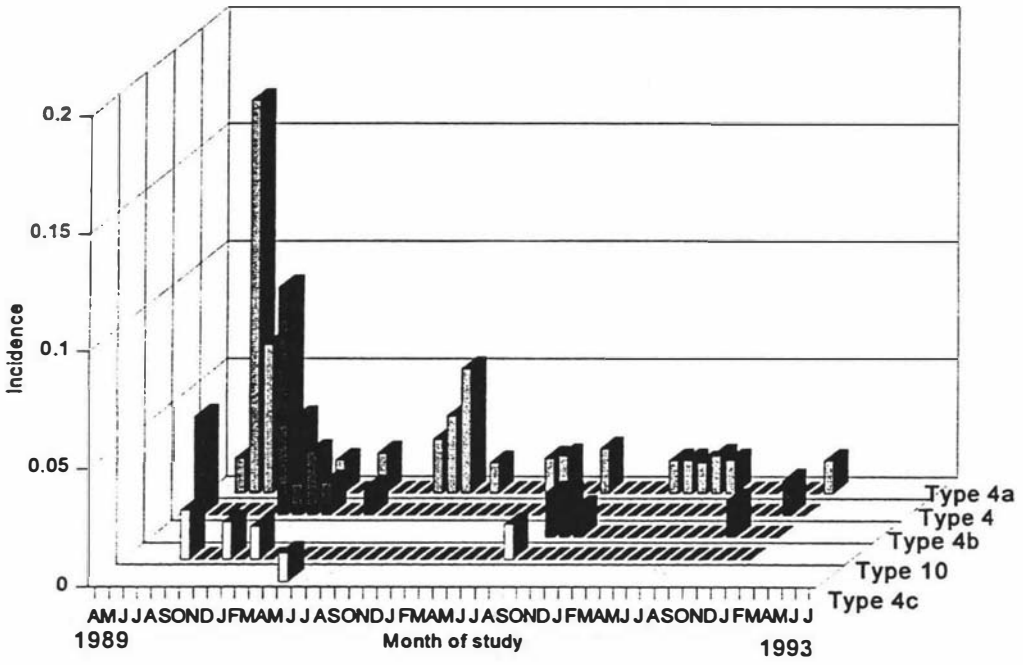


Figure 8.23. Temporal distribution of restriction endonuclease types of *Mycobacterium bovis* isolates

The locations of the 296 trap sites at the study area are shown in Figures 8.24a and 8.24b, with Figure 8.24a showing the trapsites at which tuberculous possums were caught and Figure 8.24b showing the trapsites where no tuberculous possums were recorded. It can be seen that tuberculous possums were rarely caught on the southern side of the study area.

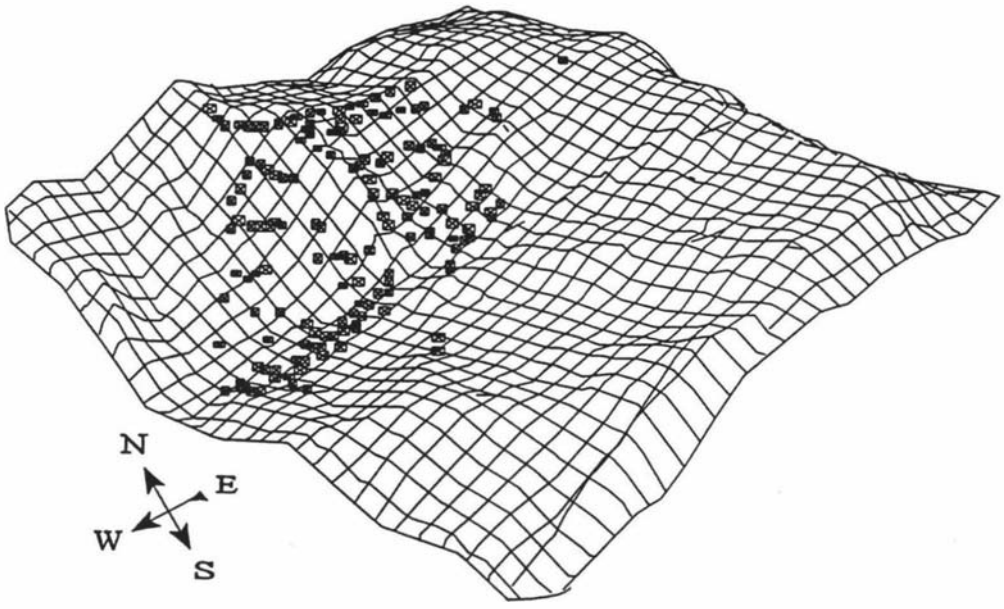


Figure 8.24a. Trap sites at which tuberculous possums were caught during the study period

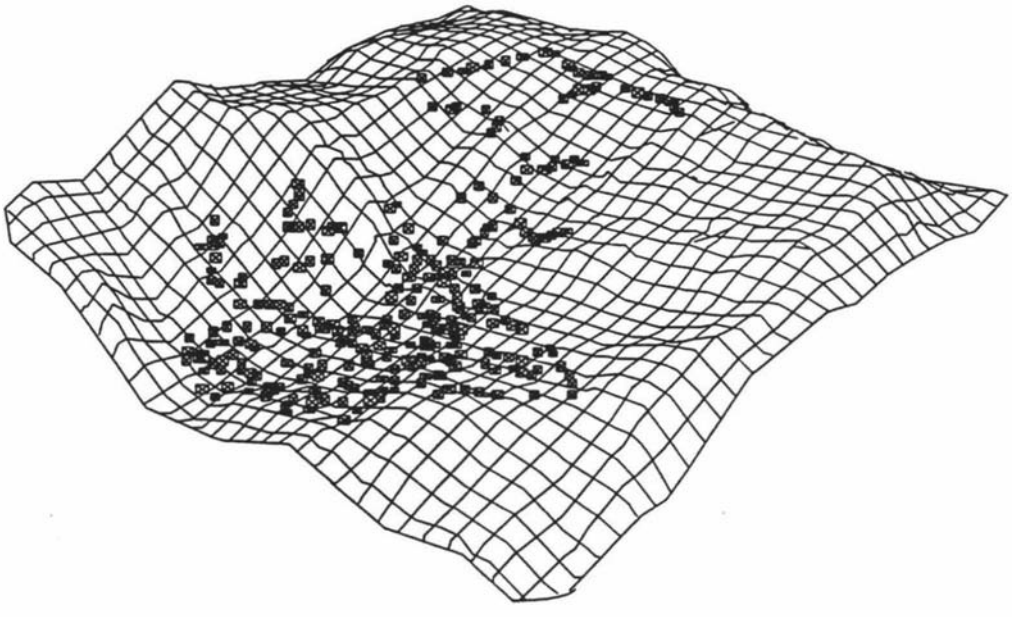


Figure 8.24b. Trap sites at which tuberculous possums were never caught during the study period

Figure 8.25 illustrates the spatial distribution of capture sites plus den-sites used by tuberculous possums infected with particular Rea types of *Mycobacterium bovis*, based on capture site data taken from the period of four months prior to time of diagnosis to time of death. This particular period was selected to improve uniformity in the data since there was wide variation in the capture histories for individual possums, arising from differences in trappability and time of entry into the study.

Figure 8.25. Spatial distribution of capture sites plus den-sites used by tuberculous possums infected with particular restriction endonuclease types of *Mycobacterium bovis* isolates, based on capture site data taken from the period of four months prior to time of diagnosis of tuberculosis to time of death

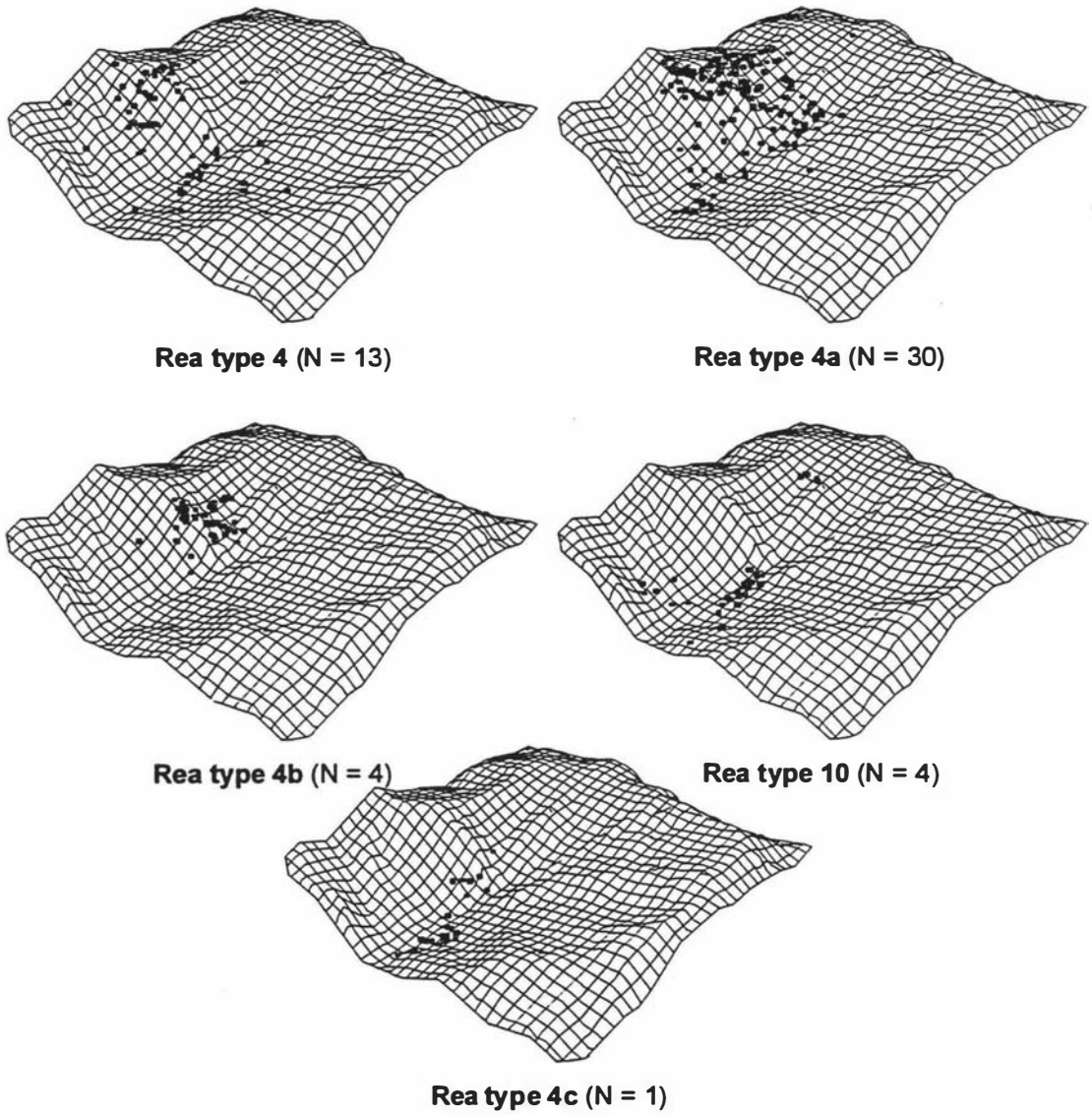
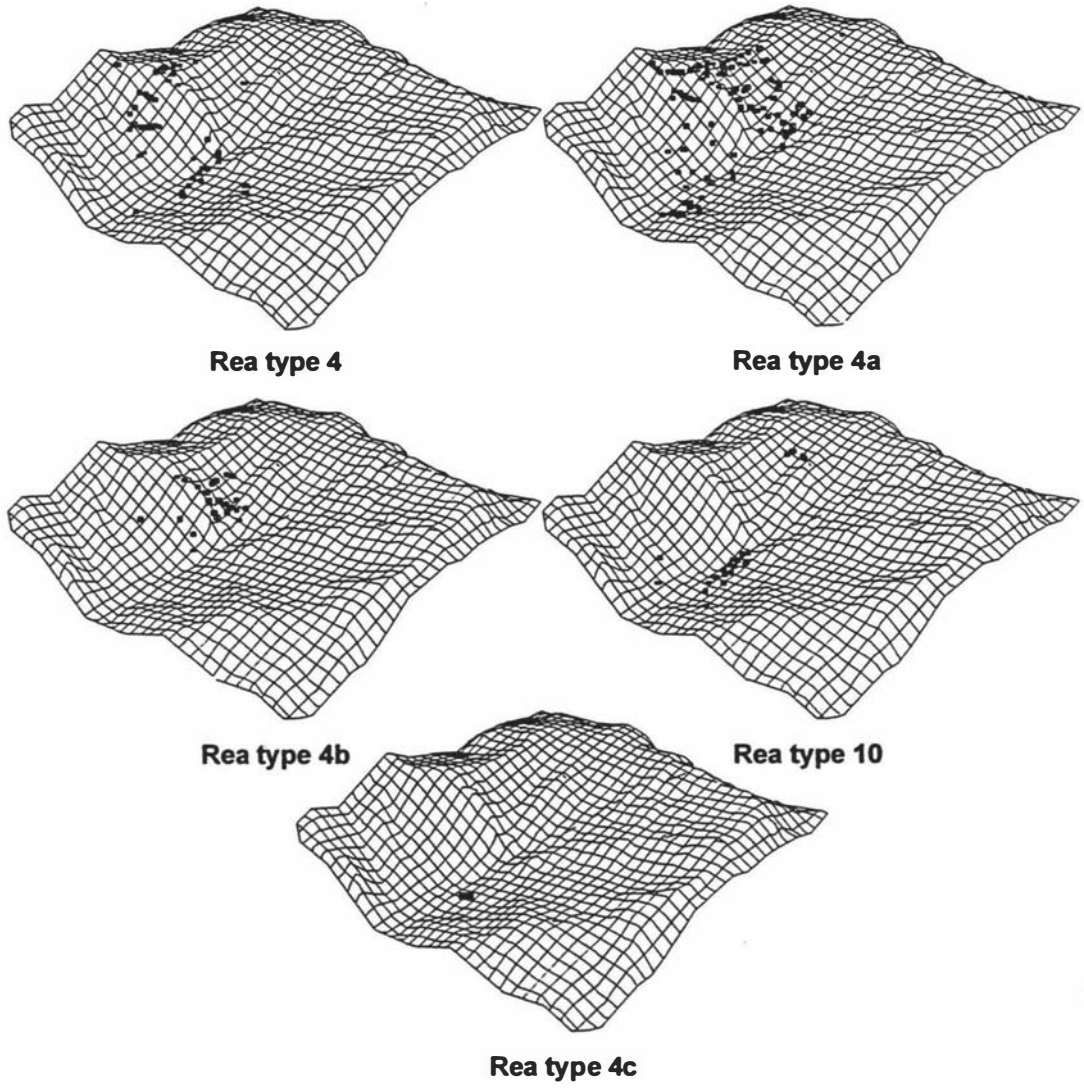


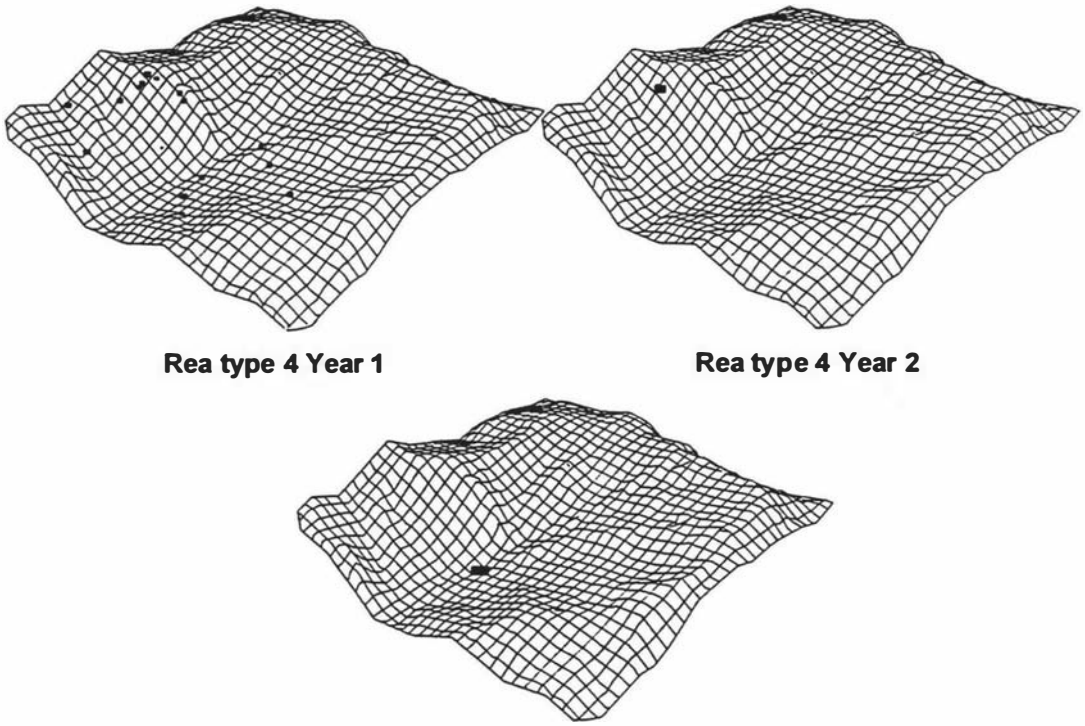
Figure 8.26 illustrates the spatial distribution of Rea types of *M. bovis* isolates based solely on capture site data from four months prior to time of diagnosis of tuberculosis to death.

Figure 8.26. Spatial distribution of restriction endonuclease types of *Mycobacterium bovis* isolates based solely on capture site data from four months prior to time of diagnosis of tuberculosis to time of death



Capture site data was used to produce Figures 8.27, 8.28, 8.29 and 8.30, which show the respective spatial distributions of Rea types 4, 4a, 4b and 10 arranged by 12 month periods. Type 4c was found during the second year of the study.

Figure 8.27. Spatial and temporal distribution of restriction endonuclease Type 4 over 52 months of the study based on locations of den-sites used by possums infected with that type



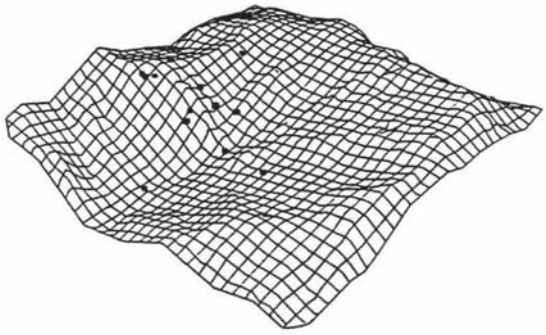
Rea type 4 Year 1

Rea type 4 Year 2

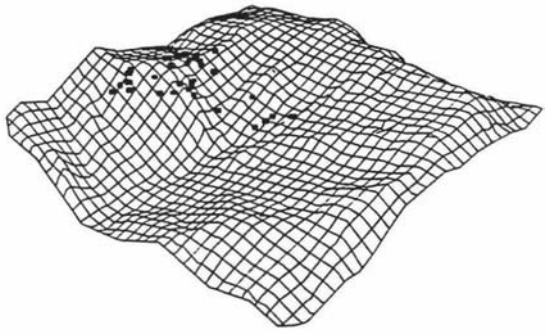
Rea type 4 Year 4

Ten cases occurred in Year 1, two in Year 2 and one in Year 4.

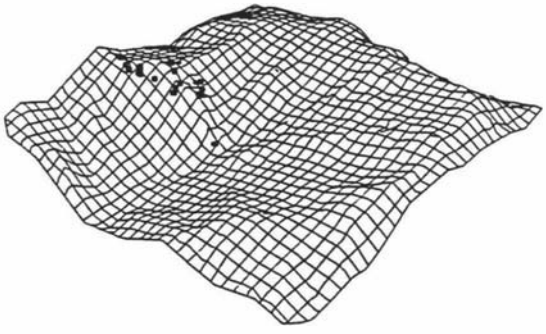
Figure 8.28. Spatial and temporal distribution of restriction endonuclease Type 4a over 52 months of the study based on locations of den-sites used by possums infected with that type



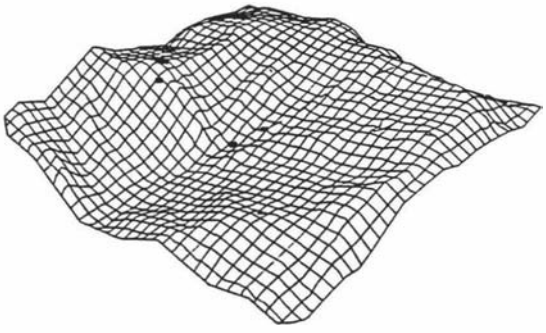
Rea type 4a Year 1



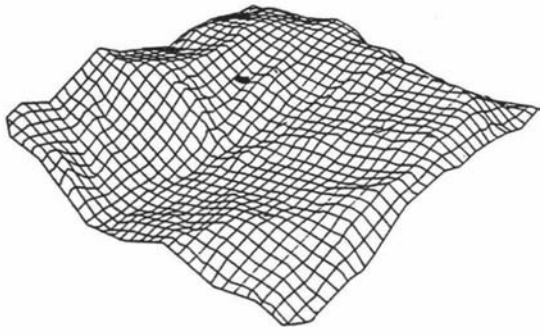
Rea type 4a Year 2



Rea type 4a Year 3



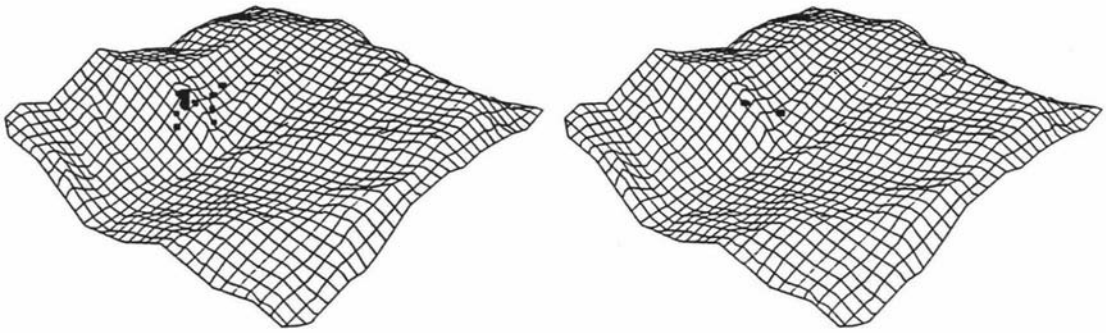
Rea type 4a Year 4



Rea type 4a Year 5

Twelve cases occurred in Year 1, nine in Year 2, three in Year 3, five in Year 4 and one in Year 5.

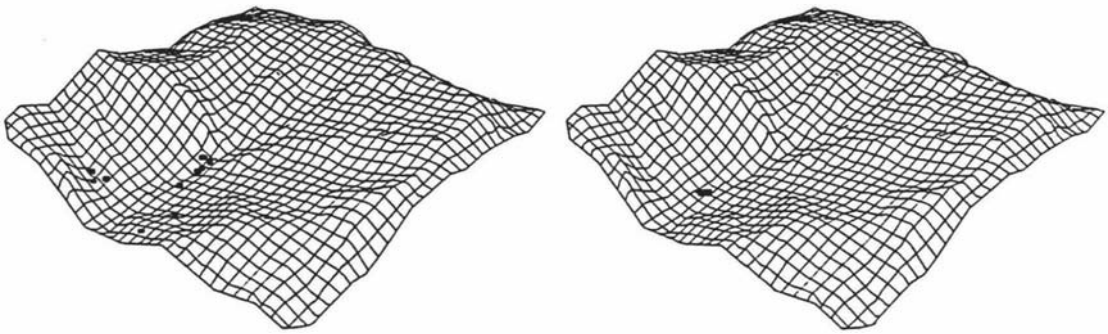
Figure 8.29. Spatial and temporal distribution of restriction endonuclease Type 4b over 52 months of the study based on locations of den-sites used by possums infected with that type



Rea type 4b Year 3 **Rea type 4b Year 4**

Three cases occurred in Year 3 and one in Year 4.

Figure 8.30 Spatial and temporal distribution of restriction endonuclease Type 10 over 52 months of the study based on locations of den-sites used by possums infected with that type



Rea type 10 Year 1 **Rea type 10 Year 3**

Three cases of this type occurred in Year 1 and one in Year 3.

TUBERCULOSIS IN OTHER ANIMALS AT CASTLEPOINT

Cattle

Groups of steers and heifers which were negative to the caudal fold intradermal tuberculin test prior to entry were grazed in Backdrop for the whole of the study period except during the summer of 1990 - 1991. In that summer Backdrop was grazed intermittently because of feed shortages and when not in Backdrop, the cattle were grazed in an adjoining paddock.

Study cattle were tested at approximately three monthly intervals. Groups were replaced on three occasions when the majority were fit for slaughter and were between 2 and 3 years of age. Thirty-one animals were introduced in April 1989 and ten of these were test positive in May 1989. All ten reactors showed lesions of tuberculosis during routine inspection at slaughter. Four of the remaining 21 tested positive in November 1989 and two of these showed lesions at slaughter. One of the remaining 17 tested positive in January 1990 but it was negative at a subsequent test in April 1990 when all were negative.

Subsequently the size of the group was reduced to between 17 and 23 animals. In mid 1990 a fence was erected in Backdrop for conservation purposes and it approximately bisected the study area and effectively restricted cattle grazing to the southern side. Thereafter no more cases of tuberculosis were found in cattle grazed on the southern side of Backdrop.

Cattle were given access to the northern side for 10 days in September 1990 and for approximately 2 weeks in November 1991, but were test negative at subsequent tests. In August 1992, four steers were put into the northern side and two of these were test positive in October 1992. At necropsy, both had disseminated lesions in the medial retropharyngeal lymph nodes from which *M. bovis*, Rea type 4a was isolated. Eight cattle were grazed in the northern side from February 1993 but there were no more reactors.

Goats

Feral goats occasionally frequented the study area. Seven were shot in September 1990 but no lesions of tuberculosis were found at necropsy.

Sheep

Twelve sheep which were living in a wild state in Backdrop were captured and shorn. Two weeks later they were tested for tuberculosis using the intradermal tuberculin test applied to the inner aspect of the thigh. No reactors were found and no lesions of tuberculosis were found at necropsy.

Ferrets

Mycobacterium bovis, Rea type 10 was isolated from the mesenteric lymph node of one of two ferrets trapped in the study area in September 1990. Another ferret was captured in December 1990 but showed no evidence of tuberculosis at necropsy and no mycobacteria were isolated from lung from this animal.

Pigs

Feral pigs occasionally frequented the study site. Two pigs, approximately 3 to 4 months of age, were shot about 300 metres from the centre of the study site. Both showed gross lesions of tuberculosis and *M. bovis*, Rea type 4 was isolated from both animals.

DISCUSSION

In the first 22 months of the study, trapping was carried out for five days each month but the effort was reduced to three days per month after that time. Trap catch success is influenced by population density, trap density and the size of the normal activity range. Trap density was high in this study with 295 traps set in fixed locations through the 21 hectare site (i.e. 14 traps per hectare) and it is unlikely that the reduction to three days per month was accompanied by any serious underestimation of population parameters.

The average trap catch success for the 52 month period was 0.248 compared to 0.21 recorded for 22 months by Pfeiffer (1994). The catch success rate increased steadily throughout the period of study and in the last six months fluctuated between 0.31 and 0.39.

At the same time, population density in the site, as calculated by the Jolly-Seber method increased. In the period from visit five to visit 50, estimated population density was 7.7 per ha and ranged between 6.4 and 9.1. The period from visit five to visit 50 was selected for reporting results of the analysis because the fifth visit coincided with the time at which trapping effort first exceeded 100 trap units, and calculations from visits 51 and 52 were dropped because end of period Jolly-Seber calculations are unreliable.

The curve for the estimated number of possums at each visit (Figure 8.8) is flatter in the latter half of the period. The lowest numbers of animals were recorded in each winter and early spring period, but the winter-early spring troughs in the curve were less pronounced in 1991 and 1992 than in 1989 and 1990.

Survival probabilities, as measured by the Jolly-Seber method are positively affected by births and immigration and negatively by deaths and emigration. Population curves need careful interpretation, because not only is it difficult to distinguish between local recruitment from births and immigration, but it is also difficult to distinguish between deaths and emigration. Although immigration might have been expected to follow the same pattern from year to year, there was an increase in the number of new possums captured during winter-early spring in the latter part of the study. Thus the flattening of the curve towards the end of the study period was influenced by immigration and possibly also by lower mortality rates in winter and early spring at that time, leading in turn to an overall increase in population density. Possums are adversely affected by

even relatively short periods of adverse cold weather and rain during which they are reluctant to forage. Death due to exposure in such conditions was commonly encountered in this study and principally affected young adolescents and animals in poor condition. Whether emigration followed a constant pattern throughout the study could not be determined.

More males than females were identified in the population, and like the proportions of immatures to matures for both sexes, this pattern was constant over time. A bias towards males can result when population expansion is caused by immigration dominated by young males, as was the case in this study. Females appeared to be more difficult to trap in the winter months during this study but the true state of the population will be more accurately defined by trapping to extinction at the end of the overall study. Coleman and Green (1984) showed unbalanced sex ratios in their live trapping study at Mt. Bryan O'Lynn but that same population showed sex parity when it was trapped to extinction.

The average at death was 23 months and was lower at 19 months for males than for females at 29 months. The estimates of average at death were biased by the greater number of yearling males than yearling females in the sample of animals for which death was recorded.

Fecundity was remarkably high, with all females examined having pouch young present in three of the five years, and high proportions (0.86 in 1989 and 0.95 in 1992) recorded in the other two years. There was no evidence of any breeding from December to February apart from three episodes in December in one year. Breeding commenced abruptly in March each year and births were recorded in every month from April to November each year. There was a secondary breeding pulse starting in each August and September although its onset was less abrupt than in the autumn. Most females in this population bred in autumn and spring. This ability is usually exhibited by colonising populations, or in localities where there are ample high quality food supplies for the lactating female and emerging pouch young.

Sexually mature males showed increased testicular tone at the times of onset of breeding activity. Some autumn born females gave birth and had rearing episodes in the following autumn but for most males sexual maturity was not reached until about 18 months of age at average body weights of 2 kg.

As in most possum populations, weaning coincided with the time of a new breeding pulse when the pouch young was about 150 days of age. Independence was apparently achieved on average at about seven months of age by which time the offspring weighed approximately 1.4 kg. The high fecundity was balanced by recorded high mortality rates in the young and a high rate of unrecorded death or emigration (which could not be distinguished between). In the cohort of 51 possums born before autumn 1992, only 10 were still known to be in the local population at the end of the 52 month period. Approximately 40% had died by that time and another 40% had either died or disappeared.

The rate of recruitment was high, with an average of 6.4 new juveniles and 2.1 new adults captured each month. The statistics for new juveniles include previously identified pouch young, but they were underestimated because several logistic reasons reduced the efficiency of identification of pouch young in this study. There was a general reluctance to attach ear tags to very small pouch young and no satisfactory alternative methods of identification were available. Re-examination under anaesthesia was carried out at minimum intervals of eight weeks, and this particular limitation, coupled with lower trappability of females in the winter, restricted the opportunities to tag pouch young. Nevertheless, the high proportion of males in the total new juvenile recruits (0.73) compared with the proportion of males in known local recruits (0.54) indicates that males dominated in immigrant juveniles, a finding which is consistant with other ecological studies of possums (Dunnet *et al.*, 1976; Winter, 1976; Clout and Efford, 1984). Adult immigration was an important component of immigration with about one quarter of all recruits first captured as adults.

Average bodyweight was consistently slightly higher for mature males than for mature females throughout most of the year. Aggregated data indicated that adult males lost weight from February-March through to July and approached parity with adult female weights in July. Females lost weight from March-April through to July and both sexes showed weight increases from November on. The adult body weight curves for males and females were remarkably similar for the whole period although female weights showed more variation in the winter period than male weights. Mean monthly body weight of mature male possums was strongly correlated with population size estimates but was not correlated with survival probabilities. Repeated measures analysis of variance showed a strong correlation between sex, season of the year, and an interaction between sex and season on adult body weights.

Although considerable tracking effort, which included the use of a fixed wing aircraft with antennae fitted to the wings on one occasion, was applied to measuring dispersal, the overall results from that effort were disappointing. Ten males and two females left the study as immatures and were tracked to, or subsequently found to have dispersed to locations, between one and 2.2 km from the centre of the study site. The study of this feature of possum behaviour was hampered by failures of radio-collars, occasional failure of receivers, and the adjacent difficult terrain to the north and west of Waio.

The information collected on denning habits was biased towards tuberculous or suspect tuberculous possums for reasons of priority and logistics. Possums favoured flax, root rakings and gorse for denning, all of which offered good protection from rain and wind. There was a strong correlation between tracking effort and the number of dens used per individual, but the effort per individual possums was relatively low and it was not possible to show whether the number of dens used per individual reached a plateau at 11 to 15 per year, as has been suggested by Cowan (1989), or was comparable to the 10 to 15 per year recorded by Green and Coleman (1987). Safe dens, well protected from the elements, are considered to be a key resource, and as such are considered to be an important element in the regulation of population density (Green, 1984). Locations suitable for dens were plentiful on the northern side of the study site and it is most unlikely that scarcity of dens produced any regulation of population density.

Den sharing outside of the mother and offspring currently being reared was very uncommon in this study and was limited to a few occasions. Pfeiffer (1994) found one possum in a den on top of the carcasses of two dead possums but although that den was subsequently regularly checked, there was no evidence of its being used again. At another location on Waio, several carcasses were found in a log hauling machine. That site continued to be used by possums, one of which was tuberculous (Lugton, pers. comm.), and additional carcasses have also since been found in it (Goile, pers. comm.). Den sharing occurs commonly in some locations in New Zealand in association with very high population densities (Fairweather *et al.*, 1987; Brockie *et al.*, 1989; Efford pers. comm.).

Sequential den use by different possums was recorded very rarely in this study.

TUBERCULOSIS EPIDEMIOLOGY

Pfeiffer (1994) discussed the relative merits of non-interventional longitudinal and cross-sectional study designs for the study of tuberculosis in possums and pointed to the clearer description of the dynamics of the disease over time and in space available from longitudinal studies. The design allows statistical assessment of possible associations between the incidence and course of the disease with spatial and temporal factors. On the other hand, longitudinal studies are expensive, require continued funding for a long period and require careful management to retain a stable and committed team of workers.

As far as is known, this study and a similarly designed study of the epidemiology of tuberculosis in badgers in England (Wilesmith, 1991) are the only non-interventional long term longitudinal studies of tuberculosis in animals to have been carried out.

Cross-sectional studies have been used to study tuberculosis in possums New Zealand, and indeed that design has formed the basis for most of the tuberculosis research in domestic animals. The cross-sectional design is simpler and less expensive but does not allow strongly based conclusions to be drawn about the temporal and spatial characteristics of the disease. The study population is distorted when necropsies are used for diagnosis, putting serious limitations on the value of any follow-up studies. In the case of tuberculosis in possums, for which there is no satisfactory test in the live animal, they have the potential for better diagnosis of disease status, depending on the degree of attention to detail during necropsies, which unfortunately has been variable in some prevalence studies.

Both designs can provide useful information, but the longitudinal design was used here with the aim of settling long standing questions about important facets of the epidemiology of the disease, and to provide statistics which could be incorporated into computerl models of the disease.

Prevalence and incidence

Calculation of prevalence and incidence statistics conformed with the criteria by Pfeiffer (1994) for numerators and denominators. Over 52 months, 9.3% of 632 possums were identified with lesions of tuberculosis (Pfeiffer, 11% of 378 possums over 22 months). Where it is appropriate in this section, comparative statistics from the 22 month period conducted by Pfeiffer are given in brackets after the abbreviation cf..

The average monthly incidence density, was 0.008 (cf. 0.014) and average monthly prevalence was 6% (cf. 8%), but monthly point prevalences varied between zero and 0.20. In the first 22 months, tuberculous animals were known to be present for all except the first visit, but during the longer study period, zero point prevalence was recorded in August and October of 1992. Given the chronic nature of the disease, it is extremely unlikely that the population was free from disease at any time. Animals were examined at two monthly intervals so that animals could have clinical disease for up to two months before detection. Furthermore clinical examination certainly underestimated the true prevalence, as evidenced by the detection at necropsy of two previously unrecognised cases which died immediately following blood collection and a negative clinical examination. Marked variation in monthly prevalence was recorded but prevalence showed a gradual decline to a low level at the end of the period under consideration.

After June 1990, tissue samples for cultural examination for mycobacteria were taken from all possums which died and were able to be necropsied in a fresh state. For the first 25 examined a piece of lung and liver were submitted for culture, but collections of pooled lymph nodes from individuals were submitted for the last 15. This change was made after it was realised that the chances of detecting small microscopic lesions in a piece of lung or liver were low compared to much higher probabilities from lymph nodes. No mycobacteria were isolated from any possums without gross lesions in the longitudinal study, but this information may have been compromised by poor selection of material for submissions early in the study.

Disease occurrence in mothers and their offspring

The risk of infection for pouch young reared by tuberculous mothers can be expected to be very high, with transmission potentially available orally via milk, via the conjunctiva, orally during regular cleaning of the pouch young and via the respiratory route. The summary histories for eight mothers and their independent offspring give qualified support to pseudovertical transmission and also serve to illustrate the frustrations which can accompany investigations of chronic diseases in free living wild animals.

In five of these mother-offspring combinations, there was cultural evidence of infection with the same Rea type. In one case there was serological evidence of pseudovertical transmission and in the remaining two cases, evidence was weak and only circumstantial. Unfortunately, the temporal sequence of infection in each combination could not be determined precisely and the evidence for and against possible alternative explanations needs careful examination.

D3513 first showed clinical disease eight months prior to a positive serological test in her female mature offspring, which was later confirmed to be tuberculous with the same Rea type as its mother. D3513 may have infected D3694 pseudovertically or they may have been infected independently either at different times or together from a common source. It is highly unlikely that a dependent offspring could be infected before its mother and that possibility is therefore ignored in further discussions on transmission between mothers and their offspring. The possibility of both becoming infected about the same time during their association together from weaning to independence cannot be discounted, but that circumstance cannot be differentiated from pseudovertical transmission. Although the course of the clinical disease in D3513 was long (9 months), and therefore the subclinical stage may also have been long, the time differences between the recognition of infection in her and D3694 are too great to strongly support pseudovertical transmission. Unfortunately D3694 did not breed, but the greatest disappointment in this particular case was the disappearance of D2910, despite her having been fitted with a radio collar.

In the case of D3709 and D3684, there was serological evidence that D3684 was diseased soon after independence although no gross lesions were found at necropsy two months later. D3709 showed serological evidence 14 months after the death of D3684 and died soon after. Assuming that D3684 was infected, she may have acquired infection either pseudovertically or together with or independently to her mother.

If D3712 was tuberculous when she reared D2270, there was no evidence that she infected D2270 who remained healthy to the end of the study period. She may have been diseased in the following year when she reared D2906, who died from tuberculosis aged 13 months. Evidence of infection was found firstly in D3712 one month after D2270 became independent at six months of age. D3712 died from tuberculosis when her offspring was 11 months old. The evidence favours pseudovertical transmission in this case, if it is assumed that both animals were diseased prior to evidence for that state. Rea types were the same in both cases.

There is strong evidence for pseudovertical transmission from D3719 to D2992. The mother, D3719, had serological evidence of infection when D2992 was seven months old and both animals had the same Rea types.

Pseudovertical transmission was almost certainly involved with D3720 and D3692 and is supported temporally and by identification of the same Rea types.

The cases of D2471 and D3728 again illustrate the frustrations and limitations associated with the study of disease in wildlife populations and incomplete information. D2471 was probably diseased when she reared D3578, which may have died or dispersed. The circumstances were reversed with loss of the mother D3728, with no evidence of infection in her but disease in her offspring. Disappearances occurred commonly in the study but both D3578 and D3728 were lost under suspicious circumstances and the possibility that both died from tuberculosis cannot be discounted.

Pseudovertical transmission occurred between D3680 and her untagged pouch young although it is uncertain whether the infected pouch young would have reached maturity in this particular case.

Although the temporal sequences in these cases did not always unequivocally support pseudovertical transmission they did not undercut it. The regular occurrence of the same Rea types in mother and offspring are supportive, but could be explained by a common source infection, or alternatively by infection acquired independently within a cluster of infection, either from possums or the environment. Potentially contaminated environmental sources include dens and food but infection involving those is discounted elsewhere in this thesis. It seems from the evidence that possums are diseased for variable periods of time before the disease is recognisable either serologically or clinically. If that circumstance commonly prevails, then the observations presented from this study would even more strongly support pseudovertical transmission.

Age and sex distribution of disease

Over the longer period of time, sexually mature animals were 8.91 times (cf. 2.25) as likely as immature animals to show evidence of disease, based on the time that the initial diagnosis was made. The marked difference between the two estimates is influenced by the increased numbers of immatures in the denominators used in calculations as the study progressed. Over both periods, the risk of showing clinical evidence of infection between males and females was the same, and there was little difference in the odds ratio statistics for likelihood of disease in mature and immature animals stratified by sex.

Caution is required when interpreting the relative risk of disease in mature and immature possums. The measures are estimates of the risk of detection of signs of clinical disease and are not representative of time of initial infection. As this and other related studies progressed, it became clear that the duration of the disease is variable and probably much longer than was previously thought. Evidence presented by Pfeiffer (1994) suggests that expression of the disease as a clinical entity may be dependent on factors which induce stressed states with subsequent lowering of immunological competence.

In the studies reported in Chapter 5, point prevalence, measured as precisely as possible by detailed necropsies, was similar in mature and immature possums, although there was a small positive effect associated with increasing age on the number of lesion sites affected, presumed to be a measure of the stage of disease. In the cross-sectional pathogenesis studies, the relative risk for tuberculosis was much higher for males, a finding which contrasts with results from this longitudinal study. The difference may relate to unequal trappability in cross-sectional studies of infected and uninfected animals of each sex.

Time of death or disease for different categories of possums

In the group of animals for which there was physical evidence of death, there was no significant difference between the age at death for tuberculous possums and possums which died from other causes, but the age at death for males was significantly less than for females in both groups of animals.

Survival analysis was used to compare the probabilities of survival of tuberculous and non-tuberculous possums. Possums which died from misadventure were removed from analyses to remove confounding from that source. Survival analysis is particularly useful for comparing survivorship among different groups, but the uncertain fate of non-affected possums which disappeared during the study needs to be taken into account when considering the presumed non-tuberculous group. Some possums may have died or dispersed, and some may have been tuberculous and not recognised as such. The true curve for the presumed non-tuberculous group lies between the curves for the non-tuberculous cohort which disappeared and the non-tuberculous cohort which included animals known to have died and animals still in the study. Possums which developed tuberculous lesions had a probability of survival of 39% (cf. 37%) at 12 months and zero probability at 30 months. Non-tuberculous possums which either stayed in the study or were

known to die had a 77% (cf. 51%) probability of survival at 12 months and a 54% probability at 52 months. The true survival probability at 12 months was somewhere between 21% and 77%.

The survivorship functions for tuberculous males and females were similar, as were the curves for non-tuberculous animals. The relative positions of the male and female curves in the tuberculous and non-tuberculous groups were remarkably similar but the significance of this particular observation is not clear.

The longer duration of this study improved the precision of Pfeiffer's (1994) estimate of the maximum survival probability of tuberculous possums, but increasingly wide confidence limits apply as the curve moves from left to right, reflecting the decreasing number of animals at risk as time progresses. Further survival analysis, which will take into account time-varying covariates such as season of the year and maturity, are planned at the end of the overall study.

Temporal dynamics of the disease

The epidemic curves for prevalence and incidence showed a gradual decline throughout the study period. Aggregated data showed low prevalences from February to June and peak prevalence in November. The prevalence patterns for males and females were similar in shape but differed in time with a lag of about three months between them. Cases in females were more prevalent from July to October, whereas cases in males were most prevalent from October to January. Incident cases in females peaked strongly in July and August, contrasting with a more prolonged peak period from July to November for males. The data suggests that incident cases in females occurred in bursts of about three months duration prior to burst of cases in males in the following few months.

Pfeiffer (1994) found an association between cumulative incidence and the ratio of monthly average minimum to average maximum daily temperature (lag one month), and total monthly rainfall (lag one month). He also established a link between average body weight in adult males (lag one month) and incidence. Analyses of associations between ecological factors which might be responsible for variation in the epidemic curves were not carried out at this stage of the study but are planned at the termination of the overall study.

Spatial dynamics of the disease

This aspect of the study, although again dealt with only descriptively, showed continued clustering of the disease in time and space as established by Pfeiffer (1994). A comparison of locations of traps which caught tuberculous or only non-tuberculous possums clearly illustrates that tuberculous possums were seldom trapped on the southern side of the study site. The main denning areas were on the northern side and in Ponga gully and possums commonly moved to the valley floor pastures and the southern side during nocturnal foraging. From the use of a combination of trap site and den-site data to plot locations on the three dimensional grid, it was apparent that Rea types occurred in relatively small and well defined areas. The Rea plots show reasonably stable clustering over time when only capture site data was used for location information, and also when each Rea type was plotted separately over time using den-site location data. These latter plots suffer from incomplete information about possums which either had no den usage recorded, or where few locations were determined through radio-tracking. The den-site locations associated with particular Rea types were favoured for plotting because they gave the most precise estimates of home range locations.

Thirteen cases of Rea type 4 occurred, ten of which were recorded in the first year of the study. The dominant type in the study was Rea type 4a, which appeared to show little shift from its area of activity established in the first two years. This persistence over time may be explained readily if a concept of persistence of long lasting subclinical disease and/or chronic latent early stage disease in individuals is accepted, in turn implying that the subtype was present continuously throughout the study period. There was a high degree of immigration into the study site and although occurrences of particular Rea types may have simply been extensions of clusters in adjacent areas or the result of infected immigrants settling in the study site, it is unlikely that either explanation applied to Rea type 4a. That hypothesis is better suited to the less frequent Rea types, and particularly for types 4b, 4c, and 10, which seemed to die out over time despite multiple attempts at establishment for types 4b, and 10.

The clustering observed here indicates that individuals become infected close to their denning areas. Possums den in a relatively small area at one end of their often elliptically shaped home range of about one to three ha (Paterson, 1993) at the study site. There was little evidence of den sharing, either concurrently or sequentially at Castlepoint, and the relatively exclusive nature of individual possum denning areas contrasts markedly with the extensive overlapping of home ranges (Paterson, 1993; Sauter, pers. comm., often observed up to 30 possums sharing pasture on

the valley floor). If transmission occurred throughout the full extent of home ranges, the spatial distribution of the disease would be expected to be much greater and show expansion. Clusters may also be influenced by environmental factors which prevent the clusters from continually expanding and shifting.

The available evidence presented elsewhere in this thesis indicates that *M. bovis* organisms survive for up to three weeks in sheltered dens in the colder parts of the year. However, sequential or concurrent den sharing was rare at Castlepoint, severely limiting opportunities for infection to be acquired from contaminated dens. The nature of the spatial distribution of the disease in the population at the study site suggests that infection is acquired close to an individual's denning area but does not provide an explanation of the mode of transmission. That subject is further explored in Chapters 5 and 6 and in the final discussion.

Tuberculosis in cattle at the study site

The incidence and prevalence of tuberculosis in the mob of cattle grazing Backdrop was very high in the early part of the study and coincided with a time of high prevalence in possums, some of which died on pasture and were potentially accessible to the cattle in their terminal stages. The possibility that some of the cattle were infected but test negative prior to entry into Backdrop cannot be entirely discounted, and some horizontal transmission may conceivably have occurred between cattle, but overall the evidence supports transmission from possums.

Shortly before those cattle were replaced by a new mob, a cattle proof, but not possum proof fence, dividing the northern and southern sides was completed. Although 28 tuberculous possums were detected between the time of entry of this group of new animals and the end of the study, no more cattle cases were detected on the southern side. Two of four cattle grazed on the northern side were caudal fold intradermal test positive in October 1992, and at necropsy in December, both had well developed disseminated caseous lesions showing early calcification in their medial retropharyngeal lymph nodes. Apart from several small granulomata in the tonsil of one animal, no other lesions were found. Rea type 4a was isolated from both animals.

No cases of tuberculosis caused by Rea type 4a were known to be present in possums at the time those cattle were grazing the area. Rea type 4b was detected on the northern side in a possum which died in September 1992 and there was a further incident case of type 4 in November and another of type 4a in December. The epidemiological issue of interest is where and by which

route did the cattle become infected. The temptation to simply attribute the source of the cattle infections to possums would have been hard to resist if Rea type 4a infections were known to be present at that time. Summary interpretations include:

> There is a lag period of about three weeks between infection and reaction to the intradermal test, and cattle may have therefore been infected before entry. On the other hand the level of "contamination" with Rea type 4a at the site either in carrier animals or in the environment was high and unconfirmed cases in possums almost certainly occurred.
>
> There is no direct evidence to support infection from a tuberculous possum.
>
> A known case of Rea type 4a died in June 1992, although another case which could not be typed died in July 1992.
>
> Lesions were found in tonsil and medial retropharyngeal lymph nodes. In the absence of lesions at other sites, such cases are customarily attributed to either oral or respiratory routes of infection, albeit arbitrarily.

Unfortunately the conclusion must be that there is insufficient evidence in this particular case to preferentially support any particular hypothesis.

A significant event which may have influenced incidence in cattle at the site was the erection of the fence which effectively barred cattle from the north side, but presented no barrier to possum movements. In the early part of the study, possums commonly died from advanced tuberculosis on pasture at the bottom of Backdrop. Some of these possums had previously denned high up on the northern side but denned at valley floor level during the terminal stages of the disease, presumably because they lacked sufficient energy to climb back up the steep slopes. The greatest opportunity for contact between cattle and terminally ill possums occurred during the first 14 months of the study, when cattle had access to the whole study site and cattle density was highest. Subsequently after erection of the fence, only five terminally ill possums were recorded for short periods on the southern side of the site. Opportunities for transmission to cattle were therefore limited during that time from that source of infection and this may explain the observed pattern of disease in cattle. The role of fencing for control of the disease in cattle has not been investigated but its possible protective value warrants further study. It appears that opportunities

for possums to infect cattle are infrequent, given the high annual incidence of tuberculosis in possums recorded during this study.

CHAPTER 9

General discussion

GENERAL DISCUSSION

Stages of tuberculosis in possums

The results from the pathogenesis studies reported in Chapter 5 allow four distinct stages of tuberculosis in possums to be proposed in a simple model of the pathogenesis of the disease. The model is not meant to be regarded as a definitive explanation of the course of the disease, but is presented here to assist the discussion with regard to various aspects of infectivity and duration of the disease.

Stage I represents the period prior to formation of gross lesions. Detection of Stage I animals is compromised by decontamination procedures, which lower the sensitivity of culture tests while the alternative technique of histopathology suffers from logistical problems of finding sparsely occurring microscopic lesions. For those reasons, estimates of Stage I animals are likely to be conservative, but in the studies summarised in Table 5.1 and Table 5.10, 8.4 % of 119 possums with no gross lesions showed evidence of infection, while the proportions of Stage I to all other stages in separate studies ranged from zero of 1 and 3, 2 of 24 (= 0.08 [95% c.i. = 0 - 0.19]), 6 of 41 (= 0.15, [95% c.i. = 0.04 - 0.25]), and 2 of 9 (= 0.22 [95% c.i. = 0 - 0.49]). Most Stage I possums had lesions in more than one site, and some showed signs of early dissemination and early generalisation. Stage I possums with lung lesions potentially excrete organisms directly via the respiratory tract or indirectly via saliva or faeces. Only one Stage I possum had evidence of intestinal infection and although no primary gut lesion was found in that animal, there was potential for excretion of mycobacteria in faeces.

Single and multiple gross lesions feature in Stage II of the disease process with increased evidence of a greater extent of dissemination and generalisation. All possums with one or more gross lesions had lesions in multiple sites. Lung lesions occurred in almost all possums in Stage II, and in some there was cultural and histopathological evidence of excretion from lung. Kidney lesions occurred, but urine cultures were all negative and the nature of the lesions was not supportive of major excretion of organisms in urine as reported in badgers (MAFF, 1979), or experimentally infected rabbits (Lurie, 1941, cited by Francis, 1958), where pyogranulomatous lesions commonly affected the renal pelvis. About one half of Stage II possums had lesions in mesenteric lymph nodes and or gastric lymphocentres and histopathology detected intestinal lesions in several animals, but there was no cultural evidence of faecal excretion.

Type II animals could potentially excrete *M. bovis* organisms via the respiratory tract, saliva, faeces, and perhaps via urine, but the bulk of evidence favoured the respiratory route as having the major potential for excretion.

By Stage III, the number of lesion sites affected by gross and by gross plus microscopic lesions had further increased, dissemination of gross lesions was more pronounced, and almost all possums had obvious generalised disease. Many had discharging fistulae at this stage, and almost all of those with fistulae had all six lobes of the lung affected. As in Stage II, there was cultural and histopathological evidence of excretion via the respiratory tract but no regular or strongly convincing evidence of heavy excretion from intestinal involvement, although AFOs were detected in the gut lumen in some, and excretion by this route was probably underestimated. However, almost all Stage III possums had mesenteric and or gastric lymph nodes affected, and of 22 with discharging fistulas, 21 had mesenteric lymph node involvement. At this stage there was more regular involvement of head and neck lymph nodes, which along with gut associated lymph nodes, appeared to be subsidiary to lung lesions. Tonsils were more commonly involved and tonsillar lesions were almost always associated with generalised disease. Adult Stage III females were highly likely to have lesions in mammary glands with histopathological evidence of excretion in milk. Excretion of mycobacteria was possible in milk, and from lung, gut, and fistulae, but there was no cultural evidence of excretion via faeces or urine.

Stage IV is associated with terminal illness and probably lasts for up to 2 months, but could often be much shorter, particularly if weather conditions were harsh and unfavourable. Behaviour was altered in the latter part of this stage and the catabolic effects of advanced disease caused progressive loss of body weight. Excretion of organisms was possible in milk and from lung, saliva, gut, and fistulae, and there was cultural evidence of excretion in urine and faeces in terminally-ill possums near to death. Stage IV animals were difficult to distinguish from Stage III possums in cross-sectional studies and could have been either under or over represented if trappability of terminally ill animals was altered. All Stage IV possums had fulminating generalised disease with large gross lesions affecting major organs, and clinically appeared weaker than normal and responded poorly to stimuli. They commonly changed their area of activity during this period, and at Castlepoint tended to use dens on the valley floor instead of returning to their usual denning area higher up in the study site. During the last couple of days of life, observed behaviour was markedly altered and terminally-ill possums were prone to

wander slowly about in the daytime in a dazed state. In the first 18 months of the longitudinal study, ten of 12 possums found dead on pasture on the valley floor were tuberculous.

Although the proportions of animals in each stage could be expected to vary markedly, the data presented in this thesis suggests that at any particular time, up to 25% of tuberculous possums were in Stage I, and except for perhaps 5-10% in Stage IV, the remainder were shared about equally between Stage II and Stage III. The distribution of the numbers of gross and total lesion sites per individual shown in Figures 5.1 and 5.2 appear to follow a diphasic distribution and may reflect separate distributions of Stage III and Stage IV animals, although no firm conclusion can be drawn on that point. Apart from Stage IV, which is distinguished by onset of weight loss, no time periods could be ascribed to any other Stage. A few of the possums for which the evidence favoured infection acquired pseudo-vertically died before 12 months of age, but others showed clinical disease (either Stage II or III) and survived for up to 22 months more. Survival analyses indicated that tuberculous possums had zero probability of survival by 30 months, although that estimate may yet increase with more observation time in the longitudinal study.

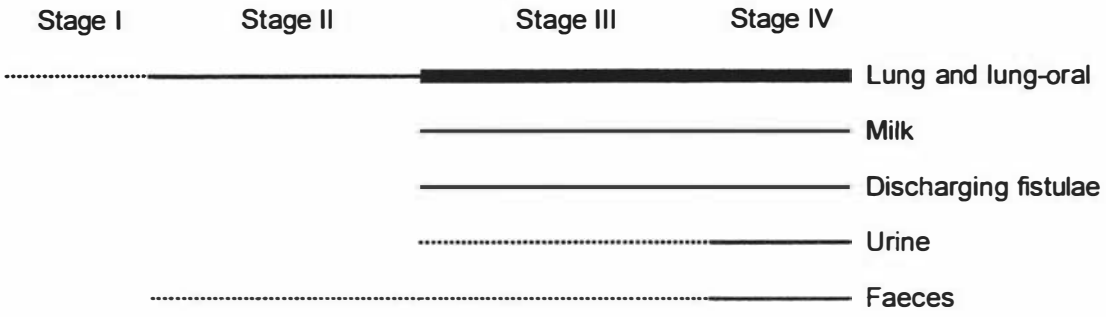
The course of the experimentally produced disease in possums has generally been rapid, and those findings may have helped to develop a perception that the course of the natural disease in the wild is also rapid. The Castlepoint experience established a long duration of natural disease, but the lack of a suitable method for determining time of initial infection means that the duration can not be calculated precisely. Close examination of some experimental infection studies (O'Hara *et al.*, 1976; Corner and Presidente, 1980) and a case study by Brockie *et al.*, (1987) also hint at a long duration of naturally acquired disease, albeit under experimental conditions for some studies. The single incident case reported by Brockie *et al.*,(1987) in the previously uninfected Orongorongo possum population may have been infected before arrival into that study area, and if that was true, it survived for a further 3½ years before dying from tuberculosis. No purposive studies have been carried out to fully investigate the course of tuberculosis in possums experimentally exposed by natural modes of transmission, and further investigation of that issue would be helpful.

Modes of transmission of tuberculosis among possums

The pathogenesis model proposes that possums become potentially infective from lung lesions soon after infection establishes in Stage I. Excretion of organisms then gradually increases from lung as disease disseminates and progresses in that organ. Excretion from other routes follows

as the disease progresses with total excretion gradually building up to the terminal illness stage, at which point there is excretion from all possible routes. The potential for the available routes at each Stage are shown in Figure 9.1, in which the thickness of the line indicates the changing level of excretion over time for that particular route. Comparison of the quantities of organisms available from each route is not possible from existing evidence and the figure is not designed to show that, but instead to allow an exploration in this discussion of the potential for transmission from each route in terms of efficiency and opportunity.

Figure 9.1. Diagrammatic representation of potential excretion of *M. bovis* organisms from tuberculous possums at each Stage of the disease process



Excretion from lung offers the greatest potential for transmission over time. In lung infections, organisms are excreted via aerosols, and in saliva, part of which is swallowed. It is not known how well *M. bovis* survives passage through the intestinal tract but faecal culture results indicated that survival was poor, despite high losses from decontamination. Investigations specifically designed to investigate survival in the intestinal tract have not been done with possums but indirect evidence from investigations in possums, other animals and man, suggest that it may be poor. In vaccine trials, Buddle (1993) found that possums vaccinated intra-tracheally or subcutaneously with BCG showed significant degrees of protection against challenge by *M. bovis*, whereas possums vaccinated via a tube into the stomach showed no protection. He postulated that the BCG was either killed in the stomach or there was immune tolerance to antigens administered by this route. Experimental studies by M'Fadyean (1910) and Chaussé (1913), summarised by Morris *et al.* (1994), showed that large doses of *M. bovis* were needed to infect animals by the oral route, indicating relative inefficiency for that route compared to the respiratory route. When discussing human infections derived from cow's milk, Francis (1958) stressed the relatively high doses thought to be needed to produce disease by the oral route in man. Working from evidence from that time when advanced disease was common in cattle, he too concluded that cattle were much more susceptible to infection by the aerogenous route than

by the alimentary route, and that despite being able to recover *M. bovis* from faeces, the epidemiological and pathological evidence indicated that infection at pasture was relatively uncommon (Francis, 1958).

Certain conditions need to prevail for successful infection via the respiratory route to occur. Key research, which established theories of airborne infection for man and animals, was reviewed by Langmuir (1961) and O'Grady and Riley (1963). Both reviews drew attention to the critical particle size needed for successful infection to occur in lung. In man, during speaking, coughing and sneezing, droplets are expelled into the immediate vicinity and either settle or dry out to become droplet nuclei which can remain suspended in air for long periods of time. From experimental infection studies in rabbits, Wells *et al.* (1948) were able to show that droplet nuclei were able to reach the alveolar surfaces of the lung. After improving their quantitative technique, it appeared that parity between the number of bacilli inhaled in droplet nuclei and the number of tubercles in the lung was reached and that a single cell would induce the development of a tubercle. On the other hand, more than 10,000 bacilli from a culture producing tubercles in the lung were ingested without observable effect. After producing more evidence in the study, they finally concluded that a few bacteria in droplet nuclei could infect, while far larger numbers of organisms in coarse particles were strained out in the upper respiratory passages and swallowed. This conclusion was supported by Lurie *et al.* (1950) who estimated that about three bacilli must be inhaled to produce a pulmonary tubercle. Particle size is critical for airborne infection. Evidence reviewed by Sonkin (1951) showed that particles above 5μ in diameter are trapped in the nose and that those smaller than 0.1μ stay suspended in the alveolar air and are ultimately again removed with the expired air. The size of tubercle bacilli is about 0.5 x 2 μ (Schlossberg 1988).

The four-year Baltimore longitudinal study described by O'Grady and Riley (1963) quantitatively investigated the infectiousness of air from a tuberculosis ward and provided convincing evidence that only very small and buoyant droplet nuclei could traverse the ducts from the ward to the animal chamber and give rise to lesions in the alveoli of test guinea pigs. They were able to identify specific patients as the source of given infections in guinea-pigs from matching of the drug susceptibility of bacilli from the patient and the guinea-pig and occupancy of the ward by the patient at the time infection of the guinea-pig was estimated to have occurred. They further showed that natural transmission of the disease could be abolished by ultra-violet irradiation of the air and that the possibility of infection being dustborne was remote.

In tuberculous possums, saliva and nasal cavity secretions are probably regularly contaminated from lung. Possums commonly chewed the bark of trunks and branches of mingimingi scrub at Castlepoint, offering opportunities for indirect transmission. No investigations of sequential bark biting activity by different possums have been carried out at that or other sites, but it probably occurs, judging from personal observations there and at other locations of very extensive marking on occasional trees, which almost certainly would have needed effort from multiple animals. Organisms deposited on trees in this manner would be available for indirect transmission by the presumably relatively inefficient oral route of infection.

Excretion via milk and from discharging fistulae commences in Stage III and continues through Stage IV. Pouch young are exposed by their mothers to infection from multiple routes of excretion which include saliva during cleaning once the eyes are open at about 90 days, aerosols, discharging fistulae and milk. Pseudovertical transmission appears to be very efficient, but it is neither possible nor necessary to determine the relative efficiencies of the separate routes which may act in that mode of transmission. Although two early cases in newly dependent possums showed evidence of respiratory infection, no satisfactory explanation could be put forward to explain distributions of lesions in other early cases. The duration of the disease in progeny of tuberculous mothers would be expected to be largely influenced by the disease state of the mother and that effect on rearing ability, the size of the infective dose and the route or routes of infection.

Organisms are potentially available for infection from discharging fistulae, principally during Stages III and IV. Although fistulae have a striking appearance, their contribution to excretion may not be great. Possums spend a considerable time each night in self-grooming and fistula orifices are kept clean, except in the last part of Stage IV, when grooming is limited by weakness. Excretion of content to contaminate the environment probably only occurs for a short time when the nodule bursts through to the exterior. The oral cavity would be infected through self-grooming from a fistula, while tree bases, branches and rims of dens may be contaminated during chesting (Winter 1976), especially if the fistula is in the axillary region. Den interiors could also be infected by discharges from fistulae. Apart from dens and sites used for chesting or territorial marking, contamination is probably slight

The contribution to transmission which urine and faeces may make is difficult to determine. There were no indications from culture tests that faeces and urine were significant vehicles for excretion until the latter part of Stage IV, although histopathology indicated that some excretion

may occur before that time. Urine marking has been described by Winter (1976), Biggins (1979) and Kean (1967), but patterns of urination outside of marking has not been investigated with the special tracking techniques and fluorescein dye as was done with badgers (Brown *et al.*, 1992). Similarly, little is known about patterns of defaecation although possum faeces could be found throughout all the study site except for within dens, where faecal matter was never seen.

Opportunities for transmission of tuberculosis among possum other than by the pseudovertical mode

Up to this point, this discussion has focused mainly on observed and potential excretion from various routes and its form and duration. The other essential element for successful transmission is opportunity, which has a strong behavioural component. The patterns illustrated in Figure 9.1 show that excretion from lung is possible for the longest time, that excretion from all routes occurs to varying extents in Stage III and that excretion is intense from most or all routes in Stage IV.

Possums are solitary animals and although the high densities which pertain in much of the New Zealand environment necessitate sharing of home ranges, possums practise mutual avoidance for most of the time. Paterson (1993) found a surprising lack of interaction between possums feeding on pasture at Castlepoint, although he reported clustering of screeching noises in the early evening and before dawn when possums were likely to come into close contact near denning areas. Simultaneous den-sharing was reported on only a couple of occasions and sequential den-sharing was rare in the longitudinal study. On several occasions, Sauter (pers. comm.) witnessed mating behaviour in which up to six males were clustered about a single oestrous female. Outside of the mother-offspring relationship, it appears that opportunities for direct transmission only regularly occur between pairs about dens and during close encounters among multiple possums during mating. More information about the frequency and nature of close contact between possums is needed. Some preliminary attempts were made to study this issue during the longitudinal study, but a prolonged effort and the use of specialised remote recording systems are required, and for the most part the attempts were unsuccessful. Direct transmission during short encounters between possums would need to be very efficient to regularly set up infection, given the limited contact time during casual encounters, although short contact times probably also apply between terminally ill possums and cattle.

Opportunities for indirect transmission occur from organisms excreted on pasture, tracks, within dens and on marked objects, but infection from those places rely on an oral route of infection. If the principles established for routes of infection in other animals and man apply equally to tuberculosis in possums, it seems likely that indirect modes of transmission produce infections only under exceptional circumstances, and therefore play a minor part in maintenance of the disease in possum populations. Furthermore, the ordered underlying pattern found in the distribution of lesions does not support infection via diverse routes, where a confused pattern of disease states and lesion distributions might be expected.

The spatial and temporal clustering of the disease described by Pfeiffer (1994) continued through Phase III of the longitudinal study, despite a lower incidence of disease in the latter part of the study. His proposal that transmission of tuberculosis between possums occurs close to denning areas and not over the whole of shared areas of activity was not refuted by the longer period of study. The amount of transmission which occurs outside of the pseudovertical mode could not be quantified but the apparent central role of denning areas focuses attention on the den itself and its particular role in transmission. Organisms appeared to survive well inside dens in the cooler parts of the year but it is highly unlikely that contaminated dens could feature in respiratory routes of infection. The floors of dens were always damp throughout the winters regularly experienced at Castlepoint, so that conditions conducive to inhalation of dust were not experienced at times of maximum survival of organisms. Furthermore, dust appears to be a poor vehicle for aerogenous infection because its relatively large particle size makes it prone to settling out in the nasal cavity or pharynx and then being swallowed.

Surprisingly little has been reported about denning habits of possums or agonistic behaviour in the vicinity of dens in New Zealand. Green and Coleman (1987) reported on den usage in a non tuberculous possum population in Westland, a region where tuberculosis is for the most part otherwise endemic. Possums in that study area denned in cavities in trees or under logs and roots, and the dens were reported as dark, confined and dusty (Coleman, 1988). In the Orongorongo valley, where tuberculosis failed to establish despite its proximity to known tuberculous possum populations, possums denned above ground in hollows in trees and in epiphytes (Ward, 1978). At Castlepoint, possums den mainly in confined sheltered dens in flax and gorse. Comparisons between denning habits of tuberculous and non-tuberculous possum populations may help to clarify the role that dens play in maintenance and spread of the disease. Taranaki possums should

be included in such a study because tuberculosis did not establish in possums there, despite initial ingredients of high prevalences in farmed stock, dense possum populations and extensive forests.

The findings from the organism survival studies and analyses of lesion distributions, when considered in the light of established knowledge of airborne spread of tuberculosis in man and other animals, leave a strong overall impression that the respiratory route should be considered to be the most important route of excretion and infection outside of pseudovertical transmission, where the relative importance of different routes could not be distinguished.

A summary of hypotheses about transmission of tuberculosis between possums

It would seem that transmission of tuberculosis between possums occurs through two major and one minor pathway. The first major pathway is pseudovertical transmission from mother to joey during the rearing process. Some infection may be transferred through milk, since the mammary gland was infected in some tuberculous animals (prevalence = 0.12) but transmission probably occurs more commonly through the prolonged and close physical contact between dam and progeny.

The second major transmission mechanism is direct horizontal transmission among adult possums with available evidence suggesting that this takes place around the locality where a possum dens, which is in effect a restricted sub-area of the more extensive home range. Possums used multiple dens located in their denning area but sequential sharing of dens was uncommon. Home ranges also overlapped extensively but the spatial clustering of tuberculosis on the study site was linked to overlapping denning areas used by particular groups of possums and was not manifested in their foraging behaviour. The most prominent opportunities for transmission occur during competition and threat/agonistic behaviour between males and during courting and mating activity between males and females. Simultaneous den-sharing except between mother and offspring was very rare at Castlepoint and could only be expected to make a minor contribution to transmission.

The third and probably least important pathway is indirect transmission among mature possums with opportunities occurring through sequential den-sharing, sequential territorial marking or through contamination of commonly shared tracks and pasture.

A plausible explanation for apparent persistence of clusters is that infection of a previously negative group of possums usually commences with the arrival of a pseudo-vertically infected juvenile animal (probably a male) which has dispersed from a pre-existing focus of infection. If this animal establishes successfully within the group and becomes infectious, it begins to produce horizontally transmitted cases in the group, particularly during the bi-annual breeding seasons. Pseudo-vertically infected juvenile females which establish successfully influence maintenance of infection within established disease clusters. Juvenile females disperse less commonly and mainly over shorter distances than males and are more likely to settle in or close to their natal home range. Whether or not there will be persistent disease depends on how rapidly each animal becomes infectious and is successful in transmitting infection to sufficient other animals for the cluster to persist despite the various factors which would cause infection to die out at that location. Multivariate analysis (Pfeiffer, 1994) has shown that spatial and temporal peaks in incidence of clinical disease were associated with circumstances likely to be stressful to possums. As proposed by Pfeiffer (1994), clustering is probably produced by two separate processes, one involving local transmission in the vicinity of den-sites and the other involving variation in the length of the incubation period, depending on the degree of environmental stress to which infected possums are exposed. The stresses associated with multiple mating occasions for males and with rearing for females plus winter environmental stress (and in particular high total rainfall over a short period) may partially explain the differences in temporal patterns of emergence of clinical disease states between males and females.

The important issues yet to be resolved are what spatial factors generate clusters and whether the location of clusters can be predicted with any degree of confidence.

Comparisons between Australia and New Zealand

Farmers and pest destruction personnel often ask why tuberculosis failed to establish in Australian possums when both Australia and New Zealand had a mix of cattle, possums and *M.. bovis* and the question is worthy of consideration in this discussion. It is not possible to give a definitive answer, but from a comparison of the ecology of possums in the two countries it is possible to produce explanations for the difference.

Now that Australia has virtually eradicated bovine tuberculosis, it is extremely unlikely that Australia will ever experience a problem with endemic tuberculosis in possums. Australia has recently declared its freedom from bovine tuberculosis although there is a residual problem in

feral buffalo in the Northern Territory. The most striking difference between possum populations in the two countries is evident in their respective population densities. In Australia, densities seldom exceed 0.5 possums per hectare, while in New Zealand, densities of eight per hectare are not uncommon. Coleman *et al.* (1980) recorded an overall density of 10.7 per hectare in a Westland podocarp broadleaf forest and a density at the forest margin of 25.4 per hectare.

High animal densities favour spread of contagious diseases such as tuberculosis, both within possums populations and between possums and cattle, because of greater opportunity for direct and indirect contact between animals. At high density areas such as the forest-pasture interface, there is an enhanced opportunity for contact between cattle and possums with increased risk of transmission of the disease. The interface between possums and cattle differs between New Zealand and Australia in terms of population density of cattle. For the most part, dry matter production of pastures in Australia is far lower than that of New Zealand cattle pastures, particularly in open eucalypt forests where the pastures are more likely to contain poorly producing indigenous species. Poor quality pastures are reflected by low population densities of cattle with reduced contact between possums and cattle and opportunity for transmission of disease. The available epidemiological evidence from Rea typing in New Zealand suggests that transmission of tuberculosis from cattle to possums occurred very rarely. Even if such an event occurred in Australia, low possum population densities would have prevailed against the disease becoming endemic in those populations. It seems likely that possums became infected in New Zealand about the early 1950's. At that time dairy farming was expanding, grazing was extending into bush margins with increased topdressing and development, and above all the prevalence of tuberculosis in dairy herds was very high. Furthermore dairy sheds and farm buildings with poor ventilation were common and they were frequently occupied by possums, providing ideal conditions for transmission from cattle to possums.

In mainland Australia, brushtail possums mainly occupy open eucalypt forests (Troughton 1954), and only in Tasmania have they successfully colonised rainforests. That difference is partly attributed to the absence of *T. caninus* from Tasmania which appears to successfully compete with *T. vulpecula* for the rainforest habitat throughout the rest of Australia (How, 1981; Bennett *et al.*, 1991). Possums are partially protected by controlled harvesting in Tasmania but the population has continued to increase there in contrast with the rest of Australia, where despite full protection, possums appear to be declining in numbers. There are no effective competitors such as *T. caninus* for possum habitat in New Zealand.

Reasons for the differences between the possum densities in Australia and New Zealand have been the subject of speculation by ecologists. Kerle (1984) concluded that availability of nutritious food was not a limiting factor for possums in the Australian environment. He pointed out that possums show remarkable flexibility in their ability to make use of varied food resources and therefore rarely suffer from lack of food in Australian habitats. He also dismissed a theory that toxins produced by eucalypts limit the intake of their leaves and pointed to the ability of possums to tolerate several toxic alkaloid containing plants and the *Gastrolobium* species which contain high levels of sodium fluoro-acetate.

Low possum population densities in Australia are most likely due to scarcity of safe den-sites. In that country, possums den above ground, commonly in hollows in trees (Mackowski 1984), to avoid predation from a variety of animals. Young animals which have recently left their mother, and young dispersing possums are at the greatest risk from predation. A scarcity of den-sites and an inability to compete with adults for this key resource puts young animals at risk when they are forced to den in sites which are accessible to predators. The persistence of adults in Australian populations supports a theory of population regulation of young animals by predators. The most important predator of possums is generally considered to be the dingo but foxes, pythons and goannas are also recognised as predators. The contrasting New Zealand environment has high possum and cattle densities with no effective predation, enhanced by periods of stress for possums associated with adolescence, mating, lactation and winter food shortages, all of which may lower the natural resistance of possums and enhance disease establishment.

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Appendix 1

TECHNIQUE FOR POST-MORTEM EXAMINATION OF POSSUMS FOR DETECTION OF TUBERCULOSIS

External examination

A unique identification is ascribed to each possum and demographic data is recorded for sex, maturity, coat colour, weight, tail length, overall body length, testes width, presence or absence of pouch young, lactation status, sex of pouch young, head length and weight of pouch young.

Prior to commencing the necropsy, the animal is examined externally for signs of swellings, trauma, and enlarged lymph nodes, which may be associated with fistulae discharging to the exterior in the parotid, submandibular, axillary or inguinal regions.

Internal examination

The possum is placed on its back with the head to the left side of the operator (for a right handed person) and a midline incision through the skin is made from the sternum to the mandibular symphysis. Incisions are then made on both sides from the manubrium sternae to a point below the elbow along the medial aspect of the forelegs.

A flap of skin is reflected down over both sides of the face to the level of the external acoustic meatus and caudally to the forelegs. The anterior neck is examined for abnormal swellings in the regions occupied by the retropharyngeal and mandibular nodes. The parotid lymph nodes which lie within the parotid salivary glands are then examined.

The midline incision is extended caudally to the perineal region. The interior of the pouch of females is examined at this stage as it is bisected. Flaps of skin are reflected from both sides of the thorax and abdomen to allow examination of the single superficial axillary lymph node and the inguinal and mammary groups of nodes. The superficial axillary node is normally small (1 to 2mm diameter) and lies subcutaneously in fascia at the level of the olecranon in the caudal part of the axilla. The inguinal and mammary chain of lymph nodes are situated subcutaneously along the inguinal groove.

The superficial and deep pectoral groups of muscles are cut at their origins along the sternum and clavicle, allowing the forelegs to be laid back and extended outwards. Care should be taken not to cut major blood vessels in the axillary space because spillage of blood makes subsequent identification and examination of the deep axillary lymph nodes difficult. The deep axillary group of nodes commonly consists of 1 large (up to 10mm) and 2 smaller nodes which are situated in fat depots in the axillary space.

The clavicles are cut at their attachment to the sternum and bent out laterally. The single superficial cervical (suprascapular) lymph nodes are situated subcutaneously and anterior to the shoulder joint and are conveniently examined from the ventral side at this stage.

The sternum and the abdominal floor are removed together by cutting along the lines of the chondro-sternal articulations and extending the cut to the pelvis. This exposes the thoracic contents and abdominal viscera and allows a preliminary inspection of those organs in situ.

The viscus is gently pulled laterally towards the right side of the animal and the chain of mesenteric lymph nodes located in the mesentery of the small intestine examined. The spleen, left kidney, renal lymph node (if enlarged) and the left adrenal can be examined in turn. A collection of gastric lymph nodes (up to 2 mm diameter), situated in fat depots adherent to the lesser curvature of the stomach and the pylorus, are examined. The hepatic node(s), situated alongside the bile duct, are conveniently examined at this stage.

If mesenteric fat measurements are required, a piece of mesentery extending from the pylorus along the greater curvature of the stomach to the spleen is stripped from its attachments to the viscera. This piece of mesentery is then weighed.

The parietal and visceral surfaces of the liver and the right kidney and right adrenal are next examined. A completely thorough examination is facilitated by removing both kidneys and the liver from the abdomen.

The thoracic organs are removed in mass from the thorax after cutting the oesophagus and posterior vena cava close to the diaphragm and then working forwards and cutting the various attachments to free the trachea and oesophagus up to the pharyngeal region. Following a careful

visual inspection of the surfaces of the lung for lesions, the lobes of the lungs are then gently and thoroughly palpated between the fingers to detect any firm nodules which may be present.

One to 3 pairs of small lymph nodes associated with the respiratory apparatus are located in the mediastinum anterior to the aorta and bounded by the brachio-cephalic trunk and the anterior vena cava.

The deep cervical (retropharyngeal) lymph nodes can be examined in situ in their location immediately caudal to the paramastoid process. The mandibular lymph nodes can be examined by cutting through the mandibular salivary gland at the angle of the mandible. The single bilaterally located single mandibular lymph nodes are situated between this gland and the angle of the mandible.

The pharynx and tonsils may be examined in situ or removed with the oesophagus, trachea and thoracic organs attached, after cutting through the symphysis of the mandible and then cutting the tissues along the medial sides of the mandibles.

Macroscopic appearance of tuberculous lesions

Small tuberculous lesions up to 4 mm in diameter are typically spherical in shape with a white to pale cream coloured exterior and commonly bulge slightly above the surface of the organ in which they are situated.. They are firm on palpation but larger lesions may be fluctuant if they have a liquid content. Lesions are seldom greater than 30 mm in diameter and proliferating large lesions may have a lobulated appearance. Lung lesions may appear to be surrounded by a thin area of hyperaemia.

Lesion contents vary from a thick creamy to a firm cheesy consistency while the colour of the content may be pale cream, pale yellow or pale lime green.

Collection of specimens for subsequent culture for *M.bovis*

Although it is not generally practicable to conduct sterile necropsies, it is possible to reduce contamination of tissues collected for culture purposes to a minimum. This may be done by strict attention to cleanliness during necropsies and the use of sterile instruments with a "no-touch" technique for collecting tissues for subsequent culture. Whole undisturbed lesions are preferred by the laboratory and sectioning of obvious lesions in the field is discouraged to reduce

contamination of the immediate working environment. A supply of sterile forceps and dissecting scissors and a container of water maintained at boiling point facilitates sterile handling of suspect lesions.

At the time of necropsy, possums are classified as grossly affected if they have caseous lesions in lymph nodes or nodules in visceral organs that measure 2 mm or more in diameter. Possums are classified as suspicious if they have enlarged lymph nodes without evidence of caseation or have nodules in visceral organs that measure less than 2 mm in diameter. Representative samples of gross lesions, usually a lymph node, are most conveniently chilled at the time of necropsy before freezing for storage prior to sending to the Central Animal Health Laboratory at Wallaceville for culture of mycobacteria.

Collection of specimens for subsequent histopathological examination

Selected tissues which show evidence of abnormalities are stored in 10% neutral buffered formalin. Whole lung may be stored satisfactorily in formalin and it is preferable to first fill the air passages of the lung with formalin introduced via the trachea with a syringe and needle.

The formalin preserved tissues are routinely processed by trimming and imbedding in paraffin blocks prior to cutting at 4 μm and staining with both haematoxylin and eosin (H & E) and Ziehl-Neelson (ZN) stains.

The second lower molar is sectioned and age determined by counting annual growth lines using the method developed by Pekelharing (1970).

Facilities for autopsies

Autopsies may be carried out in the field and in remote locations but some form of shelter for the protection of equipment and for operator comfort is essential. A source of clean water is necessary. Lighting needs to be adequate to enable detection of small lesions. A scribe to record data and organise specimen storage is desirable.

Equipment

The following equipment was used while conducting autopsies during prevalence surveys.

- Table with impervious top.
- Disposable clean rubber gloves.

- Standardised recording forms with self carbonized copy
- Water resistant tissue identification forms. Tissues are stapled to designated sites on these forms and then stored in large wide mouthed jars (1 litre Agee glass jars with sealable lids) containing neutral buffered formalin.
- Stapler and staples.
- Supply of clean newspaper. Each possum is laid on fresh sheets of newspaper which are discarded with the cadaver after each autopsy is completed.
- Butchers knives with handles capable of withstanding sterilisation in boiling water.
- Bone cutters.
- Scalpel handles and sterile blades.
- Forceps and dissecting scissors.
- Field steriliser. A large billy of water boiled over a gas ring connected to a source of gas is adequate for this purpose.
- Sterile 6ml and 20ml disposable syringes.
- Sterile disposable 16G ½" and 20G 1" hypodermic needles.
- Sterile disposable plain vacutainers and 18G 1" vacutainer needles and vacutainer needle holders.
- Sterile saline and 4" tom-cat catheters for use in collection of tracheal washes.
- Sterile disposable swabs with transport media.
- Plastic buckets to hold disinfectant solutions and fresh water for washing.
- Disinfectant.
- Soap and paper towels.
- Clean plastic 30ml specimen containers.
- 10% neutral buffered formalin.
- Insulated container with freeze packs for temporary cool storage of specimens.
- Access to a deep freeze at a nearby location for longer term storage of specimens retained for culture.
- Centrifuge, disposable pipettes and serum containers for separation and storage of sera.

Disposal of carcasses and disposable equipment

Carcasses are buried to a depth inaccessible to carrion eaters. Disinfectant solutions are discarded away from water courses. Other disposable equipment is heat sterilised prior to disposal at special locations designated by local authorities.

Protection of operators engaged in handling tuberculous possums and tissues

Operators should take care to prevent accidental self-infection with *M.bovis* as this organism may cause disease in humans. The most likely routes of infection for operators carrying out autopsies are accidental self-inoculation through open wounds or by the oral route. Operators should be aware that autopsy material is potentially infectious and precautions taken to prevent direct personal contact with organisms.

It is recommended that:

(i) gloves be worn while handling possums and conducting necropsies.

(ii) an impervious apron should be worn and contaminated clothing changed regularly.

(iii) a conscious effort be made to keep working environment clean.

(iv) instruments should be sterilised at frequent intervals.

(v) hands are washed and where possible a change of clothing be made before handling food.