LEPTOSPIROSIS IN FREE-LIVING ANIMALS

IN NEW ZEALAND, WITH PARTICULAR

REFERENCE TO THE POSSUM

(*TRICHOSURUS VULPECULA*)

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Veterinary Pathology and Public Health at Massey University.

Stephen Clive Hathaway

1978

ABSTRACT

A serological survey of 600 possums (*Trichosurus vulpecula*) from farmland environments in the southern half of the North Island of New Zealand revealed that titres against leptospires of the Hebdomadis serogroup were present in 52% of sera. Bacteriological studies identified the causative organism as *Leptospira interrogans* serovar *balcanica*. This serovar had previously been isolated only in Eastern Europe; from man, cattle and pigs.

Isolation of leptospires from kidneys was aided by the use of a system involving whole kidney homogenisation in γ-sterilised plastic bags in a 'Coleworth Stomacher'. Comparison of cultural and serological results demonstrated that, when titres were read at low minimum serum dilutions, the serological prevalence was an accurate indicator of the bacteriological prevalence.

Field surveys and experimental studies demonstrated that the possum was a typical maintenance host for *balcanica*. Experimental infection was easily established and 50% of animals were still leptospiruric one year after infection. Experimental infection with *hardjo* could not be established, and it is considered that previous reports of endemic *hardjo* infection in possums in New Zealand could be incorrect.

There was a marked difference in the age-specific prevalence of *balcanica* infection in possum populations, with infection being restricted to sexually-mature animals. Evidence is presented to support the hypothesis that infection becomes established following behavioural changes associated with the onset of sexual maturity. It is suggested that the transmission of *balcanica* within a population is dependant on direct contact between animals rather than environmental contamination. No correlation could be shown between the prevalence of infection in different populations and the nature of the habitat.

A consistent paradoxical reaction to *hardjo* was found in sera from possums infected with *balcanica*. Chromatographic studies revealed that this was due to heterologous agglutinating activity of antibodies of the IgM class. Chromatographic studies also indicated that, following

infection, the transition of the predominant agglutinating activity in sera from antibodies of the IgM class to antibodies of the IgG class was considerably slower than in eutherian mammals.

*Balcanica* was found to haemolyse red blood cells of several species. An *in vitro* haemolysin test for the differentiation of *balcanica* and *hardjo* isolates is described. Haemolytic activity has not been previously demonstrated in other members of the Hebdomadis serogroup.

Investigations of other small free-living mammals revealed that serogroup Ballum infection was endemic in ship rats (*Rattus rattus*), house mice (*Mus musculus*) and hedgehogs (*Erinaceus europaeus*) in natural and syanthropic biotopes. The Norway rat (*Rattus norvegicus*) was also shown to be capable of maintaining a focus of Ballum serogroup infection when the population density was high. No leptospires from other than the Ballum serogroup were isolated from the rodents examined.

Field evidence suggested that there was no predator-chain transmission of leptospiral infection from rodents to free-living carnivores. Birds were similarly found to be unimportant in the epidemiology of leptospirosis in the ecosystems studied.

The nidality of leptospirosis was investigated in an intensive farming environment for a period of two years, and well-defined maintenance-host parasite relationships for different serovars were defined in both domestic and free-living species. No cultural and very little serological evidence of interspecies transfer was found, despite the apparent opportunity for transfer of infection.

The concept of a maintenance host for a particular leptospiral serovar was investigated using a laboratory mouse model. As a consequence of this investigation and field studies, the characteristics of a maintenance host for a leptospiral serovar are redefined.

# ACKNOWLEDGEMENTS

Frontspiece : The Australian brushtailed
possum (*Trichosurus vulpecula*)

## TABLE OF CONTENTS

# LIST OF FIGURES

## LIST OF PLATES

CHAPTER I

LEPTOSPIROSIS IN WILDLIFE, EPIDEMIOLOGICAL CONSIDERATIONS AND A
SUMMARY OF LEPTOSPIROSIS IN NEW ZEALAND

INTRODUCTION

Leptospirosis is an important zoonosis with a world wide distribution.
Initially the disease was considered to be a sporadic infection of
rats, dogs and humans caused by a relatively small number of serovars.
More recent investigations have shown leptospirosis to be a common but
generally inapparent infection of many different species of wildlife
and domestic stock associated with a large number of different serovars.
These inapparent reservoir hosts can act as a direct or indirect source
of infection for man.

The genus *Leptospira* represents a morphologically-distinct group
of organisms of the Order Spirochaetales (Noguchi, 1918).  The type
organism, serovar *icterohaemorrhagiae,* was isolated from a human with
Weil's disease (Inada *et al*, 1916) and from Norway rats *(Rattus
norvegicus)* by Noguchi (1917).  *Leptospira interrogans* is tentatively
the only species recognised in the genus (Anon, 1967) however two
complexes, based on antigenic and biological differences, can be
differentiated; the *interrogans* complex which consists of pathogenic
strains and the *biflexa* complex which consists of mainly saprophytes.

Differentiation of leptospires within these two complexes is based
on antigenic characteristics and the *interrogans* complex is divided
into 16 serogroups, each containing strains that have shared antigens.
Individual strains within serogroups, differentiated by cross-
agglutination absorption tests, are designated as serovars.  The World
Health Organisation (Anon,1967) has defined serovars in the following
manner;  "two strains are considered to belong to different serovars
if, after cross absorption with adequate amounts of heterologus antigen,
10% or more of the homologous titre regularly remains in at least one
of the two antisera in repeated tests."   New serovars identified by
cross-agglutination absorption are continually being isolated and many
of these originate from wild animals (Anon, 1975).

As a matter of convention, the level of leptospiral agglutinins, as measured by serum agglutination tests, will henceforth be referred to as titres.

## LEPTOSPIROSIS IN WILDLIFE

Soon after the discovery that the agent of Weil's disease of man was a leptospire of the Icterohaemorrhagiae serogroup (Inada *et al*,1916) the same serovar was isolated from Norway rats (Ido *et al*,1917 ). Thus wildlife were immediately implicated as an important factor in the epidemiology of leptospirosis of man. By 1930 the Norway rat was considered to be a world-wide carrier of serogroup Icterohaemorrhagiae organisms (Uhlenhuth and Fromme,1930) and outbreaks of leptospirosis in humans were commonly associated with close contact with rats (Altava *et al*,1956; Babudieri,1958).

Other rodents were also soon discovered as carriers of leptospires. The field vole (*Microtus arvalis*) was often shown to be infected with serovar *grippotyphosa* (Borg-Petersen,1949). Epidemics of human *grippotyphosa* infection("mud fever") in Central Europe were associated with both changes in population density of field voles and climatic conditions prevailing at the time (Popp, 1950; Kathe,1950). The field mouse (*Apodemus sylvaticus*) was incriminated as a source of human *grippotyphosa* infection in the U.S.S.R. (Popova, 1957) and several other species of rodents were found to be infected with serovars associated with sporadic cases of leptospirosis in humans where man and rodent shared a common environment. These included serovars *sejroe* and *poi* in the common shrew (*Sorex araneus*) (Ananyin,1954) and serovar *pomona* in the field mouse, the house mouse (*Mus musculus*) and various species of rats (*Rattus* spp.) (Babudieri and Bianchi,1940).

Extensive investigations in the 1940's and 1950's demonstrated a large number of serovars from a wide variety of rodents. Species studied included mice (*Apodemus* spp.) (Rimpau, 1942; Kitoaka,1951; Kmety,1955; Kemenes,1957), voles (*Microtus* spp.) (Gsell,1952; Kmety, 1955; Salminen,1956; Broom and Cohlan,1958), rats (*Rattus* spp.) (Babudieri and Bianchio, 1940; Zwierz *et al*,1958), the house mouse (Das Gapta,1940; Borg-Petersen, 1944; Wolff,1954; Salminen,1956),

the common shrew (Kmety, 1955; Salminen, 1956) and harvest mice
(*Micromys* spp.) (Mino,1939; Kmety,1955; Salminen,1956).  Surveys in
tropical countries revealed new serovars in various species of
rats including serovar *bataviae* in *R.norvegicus, R diardi*, and
*R.argentiventer*, and serovar *javanica* in *R.jalorensis*  (Collier,
1948; Esseveld,1948).  The majority of these workers considered these
rodent reservoirs to be a major source of infection for both domestic
stock and man.

Reviews by Babudieri (1958) and van der Hoeden (1958), while
emphasising the importance of rodents as carriers of leptospirosis,
reported the beginning of much wider investigations of wildlife from
many Orders and species in many countries throughout the world.  This
trend has continued into the 1970's and consequently much information
has been gained on the prevalence of leptospirosis in a wide variety
of wild animals.  This greater knowledge has been of considerable
importance in assessing the role of wildlife as a source of infection
for domestic animals and man in various countries.

An important aspect of this wider understanding of the disease has
been that some domestic animals appear capable of maintaining certain
serovars, such as *hardjo* in cattle, independant of a wildlife reservoir
(Roth *et al*,1964; Hanson, 1976).

In addition to Rodentia (rats, mice, hamsters, voles, squirrels,
gerbils, coypu), leptospires have now been isolated from free-living
Insectivora (hedgehogs, moles, shrews),Carnivora (foxes, jackals,
skunks,raccoons, mongeese, feral cats),Artiodactyla (deer), Lagamorpha
(rabbits, hares), and Marsupialia (opossums, possums, bandicoots).
Sporadic isolations have also been made from non-human primates, rept-
iles, amphibians, marine mammals, birds, bats and insects.  Due to
the vast number of free-living animals capable of acting as reservoirs
of infection throughout the world, many more host-parasite associations
have been identified in wildlife compared with domestic animals and
man (Anon,1966; Anon,1975).

Within this wide spectrum of host-parasite relationships, serovar
distribution is extremely variable and, from an epidemiological point
of view, leptospirosis in wildlife can be divided into several groups
of host-parasite relationships.

1.    Some serovars have an extremely localised distribution and are host-specific.  Serovar *bakeri* of the Tarassovi serogroup has been isolated only from the Southern bush rat (*Rattus assimilis*) in the State of Queensland in Australia (Anon, 1975) and serovar *brasiliensis* has been isolated only from the opossum (*Didelphis marsupialis*) in the Sao Paulo district of Brazil (Santa Rosa *et al*, 1972).

2.    Other serovars have a localised distribution but are less host-specific.  Serovar *zazoni* of the Pyrogenes serogroup forms a well-defined localised reservoir of infection in nine species of wildlife in North Queensland (Emanuel *et al*, 1964) and is the cause of "canefields leptospirosis" in humans in this region.  Apart from this well-defined nidus, *zazoni* has also been recovered from the house rat (*R.rattus flavipectus*) in China (Chung Huei-Lan *et al*, 1960), white tailed deer (*Odocoileus virginiana*) in the U.S.A. (Roth, 1970) and man in Italy (Babudieri *et al*, 1955).  Serovar *szwajizak*of the Hebdomadis serogroup is another leptospire in this category.  This organism has been isolated from the house mouse, ship rat  (*Rattus rattus*), European hedgehog (*Erinaceus europaeus*) and man in Israel (van der Hoeden and Shenberg, 1962).  *Szwajizak* has also been recovered from the short-nosed bandicoot (*Isodoon macrourus*) and man in Australia (Smith *et al*, 1954; Emanuel *et al*, 1964) from the opossum in Brazil (Santa Rosa *et al*, 1975), from the spiny rat (*Proechinys semispinosus*) in Panama (Gale 1965) and from cattle in the U.S.A. (Glosser *et al*, 1974).

3.    Some serovars have an extremely wide geographical distribution and are capable of infecting a large number of free-living species as well as domestic animals and man.  Three serovars in this category are *copenhageni*, *ballum* and *grippotyphosa*.

Serovar *copenhageni* is commonly isolated in high prevalence from Norway rats and the worldwide distribution of this serovar in other species of wildlife, domestic animals and man is probably associated with the cosmopolitan distribution of this rodent.  This serovar has been isolated from other species of rat from different parts of the world (Lahiri, 1941; Broom, 1958; Gordon-Smith *et al*, 1961; Nityananda and Harvey, 1971) and also from the European hedgehog (Borg-Petersen

and Fennestad, 1962), opossum (Santa Rosa *et al*, 1975) house mouse
(Alexander *et al*, 1961; Minette, 1964), Indian mongoose (*Herpestes
autopunctatus*)(Minette, 1964), water vole (*Arvicola terrestris*)
(Plesko and Novakova, 1967), field mouse and several species of vole
(Parnas *et al*, 1964; Mateev, 1964). In the U.S.A., *copenhageni*
has been isolated from many species of wildlife including the Norway
rat, ship rat, house mouse, opossum, coypu (*Mycocastor coypus*), red
fox,(*Vulpes vulpes*), grey fox (*Urocyon cinereoargenteus,*) striped skunk
(*Mephites mephites*) and raccoon (*Procyon lotor*) (Roth, 1970). Infection
of domestic animals by *copenhageni*, including cattle, pigs, horses and
dogs, is well documented throughout the world (Anon, 1966; Anon, 1975).

Serovar *ballum* also has a very wide geographical distribution in a
multiplicity of hosts. First isolated from the house mouse in Denmark
(Borg-Petersen, 1944), this ubiquitous serovar has since been recovered
from many free-living animals. These include the field mouse and
Norway rat (Fraga de Azevedo *et al*, 1951), opossum (Yager *et al*, 1953),
European hedgehog (van der Hoeden, 1958), bank vole and other species
of vole (*Clethrionomys glareolus* and *Microtus* spp.) (Broom and
Cohlan, 1958), the deer mouse (*Peromyscus maniculatus*) and cotton rat
(*Sigmodon hispidus*)(Brown and Gorman, 1960), ship rat and Pacific rat
(*Rattus exulans*) (Minette, 1964) and the muskrat (*Ondatra zibethicus*)
(Paul, 1972). Serovar *ballum* has also been isolated from a reptile,
the hog-nosed snake (*Heterodon platyrhinus*) in the U.S.A. (Ferris *et al*,
1961). Despite this wide host spectrum, serovar *ballum* is most
consistently found in the house mouse from which it has been isolated
in varying prevalence from many countries. This serovar also has a
worldwide distribution in domestic animals and man (Anon, 1966; Anon,
1975) however, unlike *copenhageni*, it causes only very sporadic
infections in these hosts, even in environments where there is a high
prevalence of *ballum* infection in wildlife (Alexander *et al*, 1963;
Anon, 1965; Michna, 1970; Schnurrenberger *et al*, 1970; Brockie, 1976).

*Grippotyphosa* is a serovar that also occurs in a wide variety of
vertebrate hosts throughout the world. It has been isolated from no
less than 12 species of wildlife and cattle and pigs in the U.S.A.
(Hanson *et al*, 1964; Anon, 1966; Hanson *et al*, 1971; Anon, 1975), from
nine species of wildlife and man in Poland (Anon, 1966) and from 11
species of wildlife and man in Czechoslovakia (Anon, 1966). Many
free-living species in other countries also carry *grippotyphosa*

(Anon, 1966). This serovar has a worldwide distribution in cattle
(van der Hoeden, 1958; Michna, 1970) and it is considered that wildlife
constitute the major reservoir of infection for this species (Martin
*et al*, 1967; Fennestad and Borg-Petersen, 1972; Hanson, 1976). Conversely,
it has been suggested that in certain situations cattle may be a
possible source of infection for wildlife (Schnurrenberger *et al*, 1970).
In the U.S.A. despite the presence of *grippotyphosa* in cattle and many
species of wild animals, no human cases of leptospirosis have been
attributed to this serovar. This is in contrast to the widespread
transmission of *grippotyphosa* from rodents to humans in Europe (Popp,
1950; Kathe, 1950) and to the possible involvements of cattle in the
transmission of this serovar to humans in Israel and the U.S.S.R.
(Babudieri, 1958).

The importance of small mammals as carriers and disseminators of
leptospirosis is further demonstrated by the role of the European
hedgehog in several countries. Serovars *copenhageni, grippotyphosa,
canicola* and *szwajizak* were isolated from this species in Israel
(van der Hoeden, 1958) and serovar *bratislava* was found to be the
most common leptospire carried by the European hedgehog in Denmark
(Borg-Petersen and Fennestad, 1962). *Bratislava* was also found in high
prevalence in hedgehogs in Holland with isolates being obtained from 26%
of a sample of 125 animals (Wolff and Bohlander, 1965). These authors
considered the hedgehog to be an important reservoir host for *bratislava*
for domestic stock and man. Other workers supported this hypothesis, both
in Britain (Michna, 1970) and Denmark (Fennestad and Borg-Peterson,
1972).

Other species of small wild mammals have been shown to constitute
important local reservoirs of leptospiral infection. In Japan, several
species of voles were reported to be commonly infected with serovars
*hebdomadis* and *autumnalis*, the agents associated with "seven day fever"
and "autumn fever" respectively in Japanese farm workers (Babudieri,
1958). In Denmark a strong correlation has been shown between the
distribution of the striped field mouse (*Apodemus agrarius*) and
infection of cattle and pigs with serovar *pomona* (Fennestad and
Borg-Petersen, 1972). Fluctuations in rodent numbers were reflected

by a changing prevalence of *pomona* infection in the domestic animal
population.

Babudieri (1958) has described the harvest mouse *(Micromys minutus
sorcinus)* as an important carrier of serovar *bataviae* in Italy due
to its widespread presence in rice fields and Gordon-Smith *et al* (1961)
has suggested that the house rat *(Rattus diardi)*, a carrier of several
leptospiral serovars in Malaya, is a major source of human leptospir-
osis.

Large free-living mammals have received less attention as carriers
of leptospires. This is probably due to their fewer numbers and more
restricted distribution in enviroments shared by domestic animals and man.
However some large mammals, the most notable of which are deer, have
been shown to carry leptospires.

Most of the surveys on leptospirosis in deer have been carried out
in the U.S.A. since 1960. Serovar *pomona* was isolated from one of
31 white-tailed deer by Reilly *et al* (1962) and also from the same
species in Canada (Abdulla *et al*,1962). In a large serological survey
of deer in Wisconsin, Trainer *et al* (1963) demonstrated *pomona* titres
in 26% of 1,256 sera, and Andrews *et al* (1964), reporting the results
of a six year annual serological survey of deer, found a mean annual
prevalence of 10% of mixed titres to *pomona, grippotyphosa, canicola,*
and *ballum*. Another serological survey in the U.S.A. (Shotts and
Hayes, 1970), found titres in 292 of 1,544 white-tailed deer sera when
tested against 12 leptospiral antigens. In this survey the most
common titres were against *grippotyphosa*.

Despite the extent of serological surveys for leptospiral agglutinins
in deer in the U.S.A., very little cultural work has been attempted,
primarily due to logistical problems associated with field cultural
surveys. Hence the true prevalence of infection and the epidemiological
importance of leptospiral infections in deer have yet to be critically
determined. Experimental infection studies by Trainer *et al* (1963) in
the white-tailed deer showed that *pomona* was only excreted for a short
time in the urine although titres persisted for at least one year.
This indicated that in an endemically-infected population only a small
percentage of serological reactors would be excreting organisms.
Serological surveys of deer in the U.S.S.R.have also shown the presence

of leptospiral titres to various serovars in Japanese deer (*Cervus nippon*), (Vysotskii and Ryashchenki, 1961) and red deer (*Cervus elaphus*)(Korenberg,1975). No cultural investigations were attempted by these workers.

In contrast to the situation in the U.S.S.R. and U.S.A., deer have been shown to be only sporadically infected with leptospires in the United Kingdom (Twigg and McDiarmid,1973). These authors examined five species of deer, but found only two reactors at a minimum serum dilution of 1:30 in 345 sera and they considered that the domestic animal population constituted a risk to deer as a reservoir of leptospiral infection. A similar situation was found in Tasmania where 127 of 128 fallow deer (*Dama dama*) were found to be serologically negative. The only reactor was found to have a titre to *grippotyphosa* antigen (Munday, 1972).

Feral pigs have received some attention as carriers of leptospirosis in Australia. Keast and Littlejohns (1966) found 88 of 122 feral pigs, shot during a swine fever eradication programme, seropositive to *pomona* antigen and considered these animals an important reservoir of infection for domestic stock. A small sample of eight feral pig sera taken from an area where a local outbreak of *pomona* infection had occurred in bovines were all shown to have high titres to *pomona* (Shield, 1974). At necropsy these feral pigs had renal lesions characteristic of leptospirosis and the author considered feral pigs to be the source of this local outbreak of bovine leptospirosis. Munday (1972) found no leptospiral titres against several antigens, including *pomona*,in a small sample of feral pig sera from Tasmania.

Wild boars have been reported to carry serovar *tarassovi* in Europe (van der Hoeden,1958).

More extensive investigations have been carried out in Carnivores and in several countries foxes and jackals have been identified as leptospiral hosts. Serovar *canicola* was isolated from the Asiatic jackal (*Canis aureus*)in Israel by van der Hoeden (1955) and he considered this animal important in the epidemiology of *canicola* infections in bovines in that country. Serovars *grippotyphosa, ballum* and *icterohaemorrhagiae* have been isolated from the American red

fox (*Vulpes fulva*) and the gray fox in the U.S.A. (Roth, 1970). The
European red fox has also been found to be a host for serovar *pomona*
in the U.S.S.R., *poi* in Denmark and *bratislava* in Italy (Anon,1966)
and serological evidence of infection by several serovars in foxes in the
United Kingdom was correlated with interstitial nephritis in the kidneys
of this species (Blackmore,1964). Although leptospirosis has been
reported to occur in different species of foxes in several countries,
the significance of the disease in these animals has not been fully
determined.

The few investigations of leptospirosis in non-human primates have
revealed only a very low prevalence of serological titres, even in areas
where endemic human and rodent leptospirosis occurs (Gordon-Smith,
*et al*,1961; Minette, 1966; Minette and Shaffer,1968). Leptospiral
infection has also been found in a marine mammal. Vedros *et al*
(1971) reported high morbidity and mortality in an outbreak of
naturally-contracted leptospirosis in a herd of Californian sea lions
*(Zabophus californsonus)*. The organisms involved was subsequently
identified as serovar *pomona*, the source of which was undetermined
(McIhalton *et al*,1971).

Birds have been investigated as potential carriers of leptospires
since 1948 and there have been conflicting reports as to their role
in the epidemiology of leptospirosis. Several workers have reported
the finding of leptospiral agglutinins in the sera of birds (Gillespie
*et al*,1957; Babudieri, 1958; Twigg *et al*,1968) however attempts at
isolation of leptospires from kidneys or excreta were unsuccessul.
A claim by Addamiano *et al* (1960) to have isolated serovar *bataviae*
from wading birds has not been substantiated by subsequent workers who
have examined many species of birds, both serologically and culturally,
during investigations of endemic foci of leptospirosis in other animals
(Ferris *et al*, 1961; Torten *et al*,1965; Schnurrenberger *et al*,1970;
Munday,1972). No leptospires have been isolated in these studies.

Borg-Petersen and Fennestad (1966) found leptospires by darkfield
microscopy in the urine and kidney homogenates of 6 out of 106 mixed
species of Danish bats. The organisms could not be recovered by
conventional culture techniques or inoculation of laboratory animals
with infected material. Leptospires of unknown serogroup were also

isolated from 2 bats (*Myotis* spp) in Malaya (Gordon-Smith *et al*, 1961).
Isolates serotyped as *schnufferi* from the Canicola serogroup and
*cynopteri* from the new serogroup, Cynopteri, were isolated from local
species of bats in Indonesia (Collier and Mochtar,1939).

As is the case with birds, conflicting evidence has been presented
regarding insects as being leptospiral hosts. Some workers have raised
the question of arthropods being potential leptospiral vectors,with the
isolation of leptospires for a variable period following experimental
infection under laboratory conditions. Adult flies, *(Musca, Calliphora*
and *Lucilia* species) that had fed on infected material were shown to
harbour leptospires either in the crop or externally on the cuticle
for at least 26 hours (Kunert and Schmidtke,1952) and could therefore
possibly act as passive leptospiral carriers. A tick, *Ornithodorus
moubata*,was shown to be capable of transmitting *copenhageni* to
guinea pigs 39 days after feeding on an infected guinea pig (Schloss-
berger and Langbein,1952) and *Ornithodorus turicata* was found to
harbour leptospires for up to 232 days (Burgodorfer,1956). Three
authors reviewing the literature on leptospiral carriership by
arthropods (Babudieri,1958; Alston and Broom,1958) were of the opinion
that although under experimental conditions some insects could become
infected, no transfer to domestic animals or man had been demonstrated
and therefore arthropods were not significant carriers of leptospires.
More recent workers have isolated leptospires from naturally-infected
insects that have fed on leptospiraemic animals (Krepkogorskaia and
Rementsora,1957; van der Hoeden,1958 ) and Michna (1970) suggested
that flies may act as passive carriers of leptospirosis in the field,
however the epidemiological significance of arthropods in the
transmission of leptospirosis has not yet been determined.

The discovery of leptospiral agglutinins in snakes in Malaya
(Gordon-Smith *et al*,1961) and Florida, U.S.A. (White,1963) was the
first evidence of leptospiral infection of reptiles. Ferris *et al*
(1961) isolated serovar *ballum* from a hog-nosed snake and suggested
a predator-chain transmission of this serovar from infected mice to
snakes. Several species of reptiles have subsequently been found to
have titres to various leptospiral antigens (Andrews *et al*,1965;
Schnurrenberger *et al*,1970) and Glosser *et al* (1974) discovered a

high cultural and serological prevalence of serovar *tarassovi* infection
in several species of turtles in the U.S.A., especially *Pseudemys*
*scripta elegans*. Titres have also been reported against several
leptospiral antigens in turtles from Roumania (Combiesco *et al*,1959)
and Israel (van der Hoeden *et al*,1961). These authors reported low
titres in aquatic turtles compared with the high titres found in land
turtles, but the reason for this difference has not been determined.

The last free-living animals to be considered as hosts for lepto-
spirae are the amphibians. Diesch *et al* (1966) isolated a leptospire
from a leopard frog (*Rana pipiens*) and subsequent attempts to classify
this organism (Babudieri,1972) found it to be antigenically unique and
also non-pathogenic for laboratory animals. Babudieri (1972) also
reported the isolation of a leptospire from a toad in the Philippines.

Collectively, the epidemiological role of birds, bats, arthropods,
reptiles and amphibians in the transmission of leptospirosis to other
animals appears to be of little significance. The possibility of
some of these creatures playing a limited role in specific situations
cannot be ruled out however.

THE EPIDEMIOLOGY OF LEPTOSPIROSIS IN WILDLIFE

The epidemiology of leptospirosis in wildlife is extremely complex due to the large number of leptospiral serovars, the wide range of host species and the inconstant nature of leptospiral host-parasite relationships in a specific niche.

Several workers have conducted intensive wildlife investigations in regions where endemic leptospirosis occurs in domestic animals and man and have shown that simultaneous infection by the same serovar can occur in all three groups of vertebrates (wildlife, domestic stock and man) within the same ecological niche (Alston and Broom, 1958; van der Hoeden, 1958; Alexander *et al*, 1963; Borg-Petersen and Fennestad, 1972; Campbell and Stallman, 1975; Shenberg *et al*, 1977). Many investigations have also demonstrated infection by the same serovar in two of the three vertebrate groups present within a specific ecological niche (Kathe, 1950; Borg-Petersen and Fennestad, 1956; Babudieri, 1958; Roth *et al*, 1964; Michna and Campbell, 1970; Torten *et al*, 1970; Twigg *et al*, 1972). Other studies of endemic foci of leptospirosis however have found that wildlife was not involved as a reservoir of infection, even though wild animals of the same species may have been recognised as hosts for the specific serovar in other areas (Gillespie and Ryno, 1963; Doherty, 1967; Schnurrenberger *et al*, 1970; McCaughey and Fairlie, 1971; Munday, 1972; McDiarmid, 1975).

Where circumstantial evidence has indicated the specific role of a free-living species as a carrier and possible disseminator of leptospires, repeated observations and experimental studies have often supported the initial hypothesis (Emanuel, 1959; McGowan and Karstad, 1965; Reilly, 1970; Birnbaum *et al*, 1972; Chernukha *et al*, 1975; Kiktenko *et al*, 1976). As a consequence, many host-parasite relationships have been described in general terms of host susceptibility and infection rates but little is known about specific factors affecting interhost transmission in specific ecological niches. Some workers have stressed the importance of environment (Masaev, 1960; Ferris *et al*, 1961; Twigg *et al*, 1969; Schnurrenberger *et al*, 1970) while others have considered population density to be the most important factor affecting interhost transmission (Brown and Gorman, 1960; Andrews and Ferris, 1966). Predator-chain

transmission has also been investigated as a specific factor by Reilly,1970). The results of these investigations have been equivocal and have remained a problem of individual interpretation.

Roth (1970), reviewing the role of wildlife in the epidemiology of leptospirosis in the U.S.A., stated "current knowledge precludes an accurate assessment of the precise role of wild mammals in the maintenance of leptospires in nature and the role in the transmission of leptospirosis to man and domestic animals." This attitude can be appreciated when it is considered that the main emphasis on investigation of leptospirosis in wildlife has been on their role as a reservoir of infection for domestic stock and man rather than an investigation of the disease within the wildlife population itself. Consequently the epidemiological conclusions have often been based on small numbers of wild animals collected at a specific time during a more intensive investigation of leptospirosis in domestic stock or man. Such samples were often obtained without an understanding of the specific problems involved in determining the prevalence of lepto-spirosis in free-living populations.

The requirements for a reliable study of a disease in a wild animal population are accurate and comprehensive surveys, efficient processing of an adequate range of samples and an understanding of the biology and population dynamics of the species involved. Although the general epidemiology of leptospirosis in wild animals will be considered in depth in subsequent chapters, it is necessary at this stage to consider in general terms the requirements of field investigations that are needed to study the epidemiology of leptospirosis within a wildlife population. Such knowledge is necessary to assess the possibility of transmission of the disease to other vertebrates in the same ecological niche.

## 1. Sampling of Free-living Species

The procurement of representative samples from wildlife populations presents problems not encountered in surveys of domestic animals or man. A primary problem is the accurate assessment of the size and density of the wild life population. These factors have been shown in some studies to have significant effects on the intra- and inter-

species prevalence of leptospirosis within an ecological niche (Babudieri, 1958; Brown and Gorman,1960; Gordon-Smith *et al*, 1961c; Roth *et al*, 1963; Turner,1967). Although many methods have been employed to estimate wild-life population density (Dice,1938; Calhoun and Casby,1958; Davis,1964), most contain inherent errors and these may be compounded when different localities are sampled and compared.

The influence of specific locality on the prevalence of leptospiral infection in a particular wildlife population, both locally (Kirschner and Gray, 1951; van der Hoeden,1958; Emanuel *et al*,1964; Willis and Wannan,1966) and nationally (Parnas *et al*,1961; Wolff and Bohlander,1965; Shotts and Hayes,1970; Chernukha *et al*,1975), has been described by many workers. This variation between localities is the result of a complex interaction between many factors including population dynamics, environment, season, presence of particular leptospiral serovars and number of animal species present in the specific ecological niche. All these factors must be considered when investigating the epidemiology of leptospirosis in wildlife.

Logistical problems associated with extensive wildlife surveys and variations in trapping efficiency often affect the collection of accurate data. The animals captured may not be a representative sample of the overall population present, due to insufficient numbers being captured and differences in trapping susceptibility due to differences in behaviour associated with differences in age, sex and species.

## 2. Recording of Data

The adequate recording of data on trapped animals is of paramount importance but workers often fail to record even basic parameters such as age, sex and species. A correlation between age and prevalence of leptospirosis has been shown to occur in several free-living species (Kirschner and Gray,1951; Gordon-Smith *et al*,1961c; Wolff and Bohlander, 1965; Shotts *et al*,1975) and therefore age is a particularly important parameter to record in any epidemiological study.

Aging of wild animals is often difficult and it may only be possible to classify animals into broad categories of sexually immature and mature. Some workers have used arbitrary weight ranges as being indicative of

of age or sexual maturity (Gordon-Smith *et al*,1961c; Wolff and Bohlander, 1965; Twigg *et al*,1968; Sulzer *et al*,1968) however these are often inaccurate (Lyne and Verhagen, 1957; Calhoun, 1962; Sladen and Borg, 1969).

A different prevalence of leptospirosis related to sex has also been shown in surveys of some wild animal populations but reasons for this variation were unknown (Trainer *et al*,1963; Twigg *et al*,1972; Brockie and Till, 1977). An epidemiological study must also have accurate information on the location where animals were trapped, the correct identification of species present in the niche and the presence or absence of concurrent disease in the population being studied.

### 3. Serology

Surveys for evidence of leptospirosis in wildlife populations are often limited to serological examinations due to logistical problems associated with culturing of wild animals in the field. As leptospiral titres can only be regarded as "potentially serogroup-indicative" (Turner, 1967), serological results provide only limited epidemiological information. This cautious interpretation is necessary because of the occurrence of cross-reactions between antigenically-similar serovars and complications arising from multiple infections. Paradoxical reactions (higher titres against antigenically-heterologous serovars) may also confuse serological findings (Roth *et al*,1963; Martin *et al*,1967).

Many investigations suffer from a limited range of serovars being employed as antigens in serological tests. If an adequate range of antigens is used, some titres may be cross-reactions initiated by serovars not present among the test antigens and hence incorrect conclusions as to specific infecting leptospires can be drawn. In addition, titres to serovars not employed as antigens may be missed.

The minimum serum dilution at which leptospiral titres are read is also very variable, ranging from 1:10 (Blakelock and Allen,1956) to 1:800 (Sebek *et al*,1976). This factor must be taken into account when comparing the results from different surveys.

## 4. Culture

Any attempt to assess the leptospiral carrier status of wild animal populations must ultimately rely on culture and isolation of the infecting serovar.  Isolation of leptospires also allows definitive typing of the serovars involved in a specific nidus of endemic leptospirosis.  Isolations have often been reported from serologically negative animals (Alexander *et al*, 1963 ; Minette, 1964; Schnurrenberger *et al*,1970) and leptospires have also been recovered from animals with titres to serovars from different serogroups to the isolate itself (Roth *et al*, 1963; Martin *et al*,1967).

Materials and methods employed in cultural surveys vary considerably between different workers.  Improved media developed in recent years such as EMJH (Ellinghausen and McCullough,1965; Johnson and Harris, 1967) have been shown to be more sensitive for the isolation of leptospires compared with media used by earlier workers.

This improved cultural sensitivity allows more accurate results to be obtained, especially when serovars with fastidious growth requirements are being investigated.

Most workers have assumed that the cultural method they employed was highly sensitive for the recovery of the leptospires present in the animal population being studied.  From extrapolation of the results of the present investigation (see Chapter V), this assumption may have been invalid in some situations.  Therefore, serological and cultural prevalence ratios calculated for a population may not always be representative of the true situation.

## 5. Serovar identification

Definitive identification of leptospiral isolates by cross-agglutination absorption is an essential part of any investigation. When performed, this provides confirmation of the distribution of specific serovars within vertebrate hosts sharing a common environment, however definitive serotyping is often neglected in wildlife surveys.  In some well-defined situations, several serovars from one

serogroup have been shown to occur independantly in different free-living or domestic animal hosts sharing an ecosystem (Emanuel *et al*, 1964; Chernukcha *et al*, 1974; Mateev and Manev, 1974). In these particular situations, interspecies transmission is rare although the serovars present are similar antigentically and cannot be differentiated by serology alone.

## 6.    Changes in prevalence

Repeated sampling of animals in some wildlife populations has indicated a change in the prevalence of leptospirosis in a particular location over a period of time (Martin *et al*, 1967; Shotts *et al*, 1975; Shenberg *et al*, 1977). Although the causes of such changes may be difficult to identify, they must be considered in long-term investigations.

In conclusion, the epidemiology of leptospirosis in wildlife populations must be defined in terms of local conditions. Extrapolation from the local situation to the general is seldom valid for free-living animals.

## LEPTOSPIROSIS IN NEW ZEALAND

To date, six leptospiral serovars from five serogroups have been isolated in New Zealand (Table 1.1). These are *balcanica, ballum, copenhageni, hardjo, pomona* and *tarassovi* and are distributed amongst humans, domestic animals and wildlife.

### (i) Leptospirosis in Humans

Evidence of leptospiral infection in humans in New Zealand was first established in 1951 when Kirschner and Gray reported a titre to serovar *copenhageni* in a farmer. Serovar *pomona* was isolated from a human by Kirschner *et al* (1952) and an association between dairy farming and human leptospirosis in New Zealand was first suggested the following year by Faine and Kirschner (1953).

A survey comprising 317 human sera collected from 1952 to 1956 was tested against twenty antigens at a primary dilution of 1:300 by Josland *et al* (1957) and titres to *pomona, copenhageni, medanensis, autumnalis* and *tarassovi* were demonstrated. This provided the first evidence of human infection with a leptospire of the Hebdomadis serogroup, represented in this survey by *medanensis* antigen.

The second human serovar to be isolated in New Zealand was the hitherto unsuspected *ballum* (Anon,1967b)and Christmas *et al*(1974), investigating a local outbreak of human leptospirosis in a dairy farming region, isolated serovar *hardjo* from several farmers with clinical symptoms of leptospirosis.

Since leptospirosis was declared a notifiable disease in 1952 there has been a steady annual increase in the number of notified human cases of leptospirosis until in recent years it has become one of the most common human notifiable diseases in New Zealand. Leptospirosis is a significant occupational risk for dairy farmers and to a lesser extent other occupational groups engaged in the handling or processing of livestock (Christmas *et al*,1974; Phillip,1976). Examinations of Annual Reports of the New Zealand Department of Health for the years 1970 to 1976 reveals that Hebdomadis and Pomona serogroup infections are responsible for over 99% of human leptospirosis in this

Table 1.1 : First reports of leptospiral serovars isolated in New Zealand.

| Serogroup | Serovar | Host species | Reference |
|---|---|---|---|
| Hebdomadis | *hardjo* | human | Christmas *et al*,1974 |
| | | cattle | Lake, 1973 |
| | *"hardjo"* | possum | Brockie, 1975; de Lisle *et al*,1975 |
| | *balcanica* | possum | Marshall *et al*,1976 |
| Pomona | *pomona* | human | Kirschner *et al*, 1952 |
| | | cattle | Anon, 1951 |
| | | sheep | Anon, 1951 |
| | | cat | Harkness *et al*,1970 |
| | | pig | de Jong and Fowler,unpublished,1968 |
| | | dog | Te Punga and Bishop, 1953 |
| Icterohaemorrhagiae | *copenhageni* | Norway rat | Kirschner and Gray, 1951 |
| | | cattle | Dodd and Brackenridge, 1960 |
| Ballum | *ballum* | human | Anon, 1967b |
| | | cattle | Ris *et al*,1973 |
| | | Norway rat | Brockie, 1977 |
| | | ship rat | Brockie, 1977 |
| | | hedgehog | Brockie and Till, 1977 |
| | | house mouse | Brockie, 1977 |
| Tarassovi | *tarassovi* | pig | Ryan and Marshall, 1976 |

country.  Since 1972, *hardjo* has been the predominant serovar incriminated to human infections whereas before this date infections attributed to serovar  *pomona* were more common (Phillip,1976).

Serological evidence of infection with organisms of the Tarassovi and Icterohaemorrhagiae serogroups is occasionally found in humans, however the infecting organisms have yet to be isolated.

## (ii) Leptospirosis in domestic animals

Although a clinical syndrome attributable to leptospirosis was recognised in bovines earlier, it was not until 1950 that serovar *pomona* was isolated from cattle and sheep, so confirming the presence of leptospirosis in New Zealand domestic stock (Anon,1951).  This serovar was subsequently shown to be associated with abortion in cattle (Te Punga and Bishop,1953) and localised epidemics in sheep caused high morbidity and mortality in lambs and variable clinical disease in mature animals (Hartley,1952; Webster,1955).

Since these early reports, serovar *pomona* infection has been shown to be widespread in bovines throughout New Zealand and can cause a variety of symptoms, ranging from an asymptomatic carrier state  to mastitis, agalactia, haemoglobinuria and abortion (Anon 1973).        In calves an acute syndrome of haemoglobinuria and sudden death can occur, although young animals can also be asymptomatic carriers (Salisbury,1954; Jamieson *et al*, 1970).

The pig was recognised as a reservoir of endemic *pomona* infection in New Zealand by Salisbury (1954) and subsequent investigations have confirmed a high prevalence of this leptospire in this species (Russell and Hansen,1958; Ryan and Marshall,1976; Hodges,1977;Ryan,1978). Infections with serovar *pomona* also occur in other domestic animals and the organism has been isolated from the dog (Salisbury ,1954) and cat (Harkness  *et al*,1970).

The isolation of serovar *hardjo* from a healthy calf by Lake (1973) provided the first evidence of what has subsequently proven to be endemic leptospiral infection due to the Hebdomadis serogroup in

New Zealand cattle (Anon, 1974a; 1974b; Brockie, 1976; Hellstrom, 1978). This reservoir of Hebdomadis infection has been closely associated with a high prevalence of Hebdomadis serogroup infections in humans in this country. The disease is especially common in dairy cattle in spring and early summer in years of high rainfall (Anon, 1973) and this high prevalence is correlated with peaks of human infection at these times (Brockie, 1976; Phillip, 1976).

No definite pathogenic effects have been associated with serovar *hardjo* infection in cattle in New Zealand, which is in contrast to reports from other countries (Sulzer *et al*, 1964; Sullivan and Callan, 1970; Hoare and Claxton, 1972; Johnson *et al*, 1974; Ellis and Michna, 1976; Gordon, 1977). These workers have associated serovar *hardjo* infection with a mild clinical syndrome which can be manifested by mastitis and occasionally abortion. The organism has yet to be recovered from an aborted foetus and definitely typed by cross-agglutination absorption, however, in 1977, Ellis and Michna produced abortion in experimentally infected heifers with an Hebdomadis serogroup isolate that was very similar antigenically to serovar *hardjo*.

Titres to Hebdomadis serogroup antigens have been demonstrated in horses (Anon, 1974b; I. Doe, pers. comm.) and sheep (unpublished data). The nationwide prevalence and epidemiological significance of these titres is unknown.

Two other serovars were shown to infect cattle in New Zealand with the isolation of *copenhageni* from calves by Dodd and Brackenridge (1960) and the isolation of *ballum* and *copenhageni* from apparently healthy calves in the Hauraki Plains by Ris *et al* (1973). *Copenhageni* is capable of producing high morbidity and mortality in susceptible animals (Dodd and Brackenridge, 1960). *Ballum* has been associated with photosensitisation (Anon, 1976) and has also been isolated from a fatal infection in a calf which was characterised by haematuria and nephritis (Anon, 1977b).

Titres against serovars *ballum* and *copenhageni* have also been reported to occur sporadically in pigs (Kirschner *et al*, 1952; Ryan, 1978) but no isolations have been made to confirm infection by these serovars.

In the preliminary screening of a large laboratory animal colony the author demonstrated widespread,unsuspected *ballum* infection in adult laboratory mice. This situation has been shown to exist in experimental mouse colonies in other countries (Babudieri,1958) and has important human health implications.

The serological indications of *tarassovi* infection in earlier surveys of pigs were confirmed by the isolation of *tarassovi* from a commercial piggery by Ryan and Marshall (1976). Since then this serovar has been demonstrated in pigs from several localities in the North Island (Ryan, 1978),although at a much lower prevalence than *pomona*. Titres to serovar *tarassovi* have also been found in bovines (Hellstrom,1978).

Several workers have investigated the serological prevalence of canine leptospirosis in New Zealand. An early report of titres against serovar *canicola* in three of ten dogs from Dunedin (Kirschner and Gray, 1951) has not been supported by subsequent investigations (Salisbury, 1954; Jamieson *et al*,1970). The apparent absence of Canicola and Icterohaemorrhagiae serogroup infections in dogs in New Zealand is remarkable considering the worldwide distribution of these infections in canines (van der Hoeden, 1958; Michna, 1970; Kaufman,1976).

Routine screening of large numbers of bovine sera at government laboratories have produced numerous reports of leptospiral titres against serovars other than those mentioned above (Anon,1974a; 1974b; Anon,1976). No consistent results have been obtained however and consequently there is no evidence of widespread leptospiral infection due to an undiscovered serogroup in bovines in this country. A similar situation exists for other domestic livestock, however most species have not been thoroughly investigated.

With the presence of four serovars established in the New Zealand cattle population it is important to have an understanding of the relative prevalence of leptospiral infection attributed to these different leptospires (Table 1.2). This relative prevalence parallels that which occurs in human leptospirosis in this country. It can be seen that Hebdomadis serogroup infections are the most common in cattle, as is the case in humans. Serogroup Pomona infections play a lesser role and titres against Ballum and Icterohaemorrhagiae serogroup antigens occur in less than 1% of sera tested.

Table 1.2 :Percentage distribution of leptospiral titres against four antigens in cattle and humans in New Zealand.

| Reference | No.cattle tested | % positive at serum dilution 1:200 | | | |
|---|---|---|---|---|---|
| | | Hardjo | Pomona | Copenhageni | Ballum |
| Lake,1973 | 890[a] | 18 | 5 | 0.4 | 0 |
| Ris *et al*, 1973 | 208 | NT[b] | NT | 1.5 | 2.5 |
| Anon,1974a | 12888 | 11 | 2.5 | 0.1 | 0.02 |
| Anon,1974b | 250 | 21 | 9 | 0.4 | 0 |
| deLisle *et al*, 1975 | 77 | 51 | 0 | 0 | 0 |
| | No. humans tested | | | | |
| Christmas *et al*,1974 | 47[c] | 47 | 28 | 0 | 0 |

[a] sera tested at 1:100 minimum dilution

[b] NT = Not Tested

[c] from presumptive cases of leptospirosis. Serological prevalences quoted by Brockie, 1976.

(iii) Leptospirosis in Wildlife

There have been few systematic surveys of wildlife for leptospirosis
in New Zealand.  Most investigations have involved small numbers of
wild animals and results have been limited by inadequate serological
and cultural examinations of field samples (Table i.3).  This
situation was partially rectified by the recent work of Brockie,(1975,
1976 and 1977) however much still remains unknown about the prevalence,
geographical distribution and epidemiological importance of leptospirosis
in wildlife in this country.

Preliminary studies of the Norway rat by Kirschner and Gray (1951)
were influenced by the role of this rodent in other parts of the
world as a carrier of serovar *copenhageni*.  One hundred rats were
collected from the cities of Auckland, Christchurch and Dunedin
and sera were tested in a macroscopic tube agglutination test against
a battery of four leptospiral antigens, *viz.*, *copenhageni*, *canicola*,
*bataviae* and *australis*.  A sterile scraping with a platinum loop was
taken from the cut surface of the renal cortex and inoculated into
Gardner's medium enriched with guinea pig serum.  The sex and approx-
imate age of the rats processed were recorded.  Positive titres against
*copenhageni*  antigen were obtained from 8 of 53 Norway rats (15%) and
isolates were cultured from 2 of 53 animals.  Both of these isolates
were obtained from only one of two kidneys cultured from each rat.
It is likely that both kidneys are infected in an animal carrying
leptospires and therefore the isolation of organisms from only one
kidney demonstrates the limitations of the cultural technique used.
The serogroup only of two isolates was determined by titration
against rabbit *copenhageni* antisera, however both isolates were
reported as serovar *copenhageni*.  No positive sera or cultures were
found in 47 ship rats from the three cities.

Titres against serovar *copenhageni* antigen were found in rats
from Auckland and Dunedin, but not from the three rats caught in
Christchurch.  Isolates were obtained from only the Dunedin rats.

Salisbury (1954), in a study of Pomona serogroup infection in New
Zealand domestic animals, found no titres against *pomona* antigen
in 56 possum sera.  No cultures were attempted and no details as
to whether other antigens were used in the serological screen were

given. Similarly, no information on primary serum dilution, the serological method employed or the geographical origin of the possums was given.

Leptospirosis in hedgehogs *(Erinaceus europaeus)*, attributed to serovar *pomona* infection, was reported by Webster (1957). This author found leptospiuria in one of two hedgehogs from a Manawatu dairy farm on which bovine leptospirosis had been diagnosed. Titres against *pomona* antigen were found in sera from both hedgehogs and Webster concluded that these animals were capable of carrying serovar *pomona* and hence may have played a role in the dissemination of this organism. Cultural isolation and identification of the infecting organism were not attempted.

A survey of rats, presumably Norway rats, from the South Auckland area (Shortridge, 1960) revealed "positive or suspicious" titres against *copenhageni* antigen in 8 of 75 sera tested (11%). No cultures were attempted and there were no details as to the serological procedure used or the utilization of other leptospiral antigens. Dodd and Brackenridge (1960) also mentioned examining a "few" rats, of unidentified species, for evidence of leptospirosis in the South Auckland region. No details of methodology were given but negative results were reported.

A more comprehensive serological survey of rats from in and around Wellington city, provided no evidence of leptospiral infection in 121 ship rats and 62 Norway rats (Blakelock and Allen, 1956). Serovars *pomona, canicola, australis* and *hyos* were employed as antigens at a low initial serum dilution of 1:10. The rats were trapped from various city environments including foreign ships, docks, meatworks and offices.

Further studies of wildlife from urban environments were made by Smith (1964) with the serological screening for leptospirosis of 104 hedgehogs from Auckland, Hamilton, Uppper Hutt and Dunedin. Three positive sera were found, one with a titre to *copenhageni* and two with titres to "Pool 1" antigens which included *copenhageni, canicola* and *ballum*. All three positive sera were from hedgehogs captured in Hamilton.

The first report of an investigation into the possibility of leptospirosis in large wild animals in New Zealand was a brief note (Smith, 1965) describing the absence of titres in 15 deer sera. No details as to geographical origin of the samples, antigens employed or species of deer were given. In the same report Smith also mentioned the finding of leptospiral titres in 2 of 98 hedgehogs. Similarly, no technical details were given however it was reported that 11 animals were cultured with negative results.

A sample of 102 sera from red deer (*Cervus elaphus*) shot in the Kaingaroa Forest in central North Island was examined by Daniel(1966). Data on sex, age, general body condition and location where the animals were shot was recorded and this was the first attempt since Kirschner and Gray (1951) to undertake a systematic analysis of the animals involved in the survey. Serovars representing seven serogroups were used in the microscopic agglutination test (MAT), *viz.*, *pomona*, *canicola*, *copenhageni*, *grippotyphosa*, *australis*, *tarassovi* and *andamana*. Titres were read at serum dilutions of 1:10 and 1:200 and one reaction to *pomona* antigen was found at 1:200 in the sera of one adult hind. This animal was shot close to a farming area where there had been outbreaks of bovine leptospirosis due to *pomona* twelve months earlier. A suspicious titre to *andamana* antigen at 1:200 was also found and there were a total of eleven positive reactions at the 1:10 serum dilution against *copenhageni (3), andamana (3), pomona (4)* and *tarassovi (1)*. These low titres were regarded as non-significant by the author and he concluded that red deer were unimportant in the epidemiology of leptospirosis in domestic animals in New Zealand.

In another study, Daniel(1967) examined 393 sera from seven species of wild ungulates. Serological methods and recording of data were the same as in the 1966 survey. The sample consisted of 150 feral goats, 36 feral pigs, 93 Himalayan thar (*Hemitragus jemlahicus*), 14 chamois (*Rupicapra rupicapra*) and 170 deer of several species, (red, fallow, *Dama dama*,) white-tailed and Japanese deer) These animals were shot throughout New Zealand in localities where there was varying contact with domestic stock. Only one titre at the 1:200 serum dilution was recorded. This was from a feral goat serum reacting to *pomona* antigen. Four positive reactions at 1:10 were recorded against *tarassovi* antigen, of which three were in feral goats and one in Japanese deer. These low titres were regarded as non-significant

by the author, and he concluded, as for red deer, that introduced
wild ungulates did not play a significant role in the epidemiology of
leptospirosis in New Zealand.

Brief mention was made of a limited number of possum sera being
examined by Jamieson *et al* (1970), all of which were reported as
negative. No details were given as to antigens used or number of
possums tested but it is probable that these sera were from a sample
trapped at Murchison on the West Coast (B.Cook, pers. comm.).

An epidemiological investigation of leptospirosis at an artificial
breeding centre in the Manawatu (Blackmore *et al*,1976) included the
serological and cultural examination of a number of species of wildlife
present on the property. Test antigens were *pomona, copenhageni, hardjo*
and *ballum* and in addition some sera were also tested against *australis,
bataviae, canicola, grippotyphosa* and *tarassovi* antigens. Kidneys were
cultured by forcing a piece of cortex from each animal through a syringe
and inoculating the homogenate into Stuarts, Fletchers and EMJH media.
Seven species of wild animals were caught; 17 rabbits *(Onycytolagus
cuniculus)*, 5 hares *(Lepus europaeus)*,3 possums, 4 hedgehogs, 2 Norway
rats, 1 ship rat and 1 ferret *(Mustela putoris)*. Positive serological
titres against *ballum* antigen were found in 1 rabbit (serum dilution
1:256) and 1 Norway rat (serum dilution 1:32). Isolates belonging
to the *ballum* serogroup were obtained from both these animals. In
addition, low titres(1:200 serum dilution) were found against *copenhageni*
antigen in a hedgehog and a rabbit and against *hardjo* antigen in a
Norway rat and a hedgehog. Due to the lack of evidence of Pomona and
Hebdomadis serogroup infections in these wild animals the authors were
of the opinion that these infections in the cattle at the artificial
breeding centre were not introduced by a wildlife vector. It was
suggested however that low titres against *copenhageni* and *ballum*
antigens found in a small percentage of bovine sera from the farm may
have been due to an environment contaminated by wildlife carriers.

In an epidemiological study of endemic leptospirosis infection in swine
due to *pomona* (Buddle and Hodges,1977), 15 rats (presumably Norway
rats) and 1 hedgehog caught in the vicinity of the piggery were
examined serologically and culturally for leptospirosis. No titres
were recorded against *pomona,hardjo,copenhageni,ballum* and *tarassovi*

antigens at a serum dilution of 1:100 and kidney tissue inoculated into Fletcher's medium yielded no leptospires. It is interesting that even in an environment heavily contaminated with *pomona* organisms shed by leptospiruric swine, rats showed no evidence of infection with this serovar.

Two investigations in 1975 produced the first evidence of Hebdomadis serogroup infections in wildlife in New Zealand. De Lisle *et al* (1975), after discovering leptospires in the urine of experimental possums, subjected 26 sera from wild possums to the MAT at a minimum dilution of 1:200, using *hardjo, pomona, copenhageni* and *ballum* as antigens. Positive titres against *hardjo* were found in 17 sera (65%) and isolates were recovered from the blood, urine and/or kidneys of 5 animals. The leptospires isolated were typed by fluorescent antibody serotyping (Hodges and Ekdahl,1973) as being serovar *hardjo*.

Histopathological lesions in renal cortices attributed to leptospiral infection were also described. Titres to serovar *hardjo* antigen were also found in 39 of 77 cattle (51%) on the four farms in the Wanganui district from which the possums were taken and, based on these preliminary findings, the authors suggested that the possum could act as a reservoir of *hardjo* infection for bovines in New Zealand. This study was the first in which a *hardjo* antigen had been included in the serological screening of a significant number of possum sera.

A concurrent investigation by Brockie (1975) produced similar results. Of 146 possums sampled, mainly from dairying areas throughout the North Island, 38 (26%) revealed titres to *hardjo* antigen at a serum dilution of 1:100. From a range of seven other antigens used in the MAT (*copenhageni, tarassovi, ballum, autumnalis, canicola, pomona* and *bratislava)*, two titres of 1:100 were recorded against *ballum* and *autumnalis*.

Brockie attempted to isolate leptospires from possum kidneys by macerating the medulla in a Griffiths tube and inoculating the homogenate into EMJH media containing antibiotics. This cultural method had severe limitations (see Chapter V) and isolates were obtained from only 2 of 146 kidneys cultured. These were provisionally identified as serovar *hardjo* but neither isolate was subjected to cross-agglutination absorption tests. Brockie (1975) also suggested

that possums may play a significant role in the epidemiology of bovine leptospirosis in New Zealand.

Further work by Brockie and Till (1977) established the presence of serovar *ballum* infection in hedgehogs in New Zealand. Of 78 hedgehogs collected from widely separated areas in the North Island, five (6%) yielded isolates of this serovar. Four of these were typed at the WHO Reference Laboratory in Brisbane, Australia as being serovar *ballum*. These isolates represented the first definitive serotyping of leptospires cultured from wildlife in New Zealand. All culturally-positive hedgehogs were taken from dairy farms and 56% of this group had titres against a battery of eight antigens. The sera from 16 of 78 hedgehogs give multiple reactions to two or more antigens, and one serum had a titre against seven antigens. The authors suggested that hedgehogs are a major reservoir of serovar *ballum* infection in dairying districts.

A survey of 162 rodents from various dairy farming areas and country rubbish tips in the North Island (Brockie, 1977) revealed further evidence of leptospiral infection in New Zealand wildlife. Intensive trapping on dairy farms resulted in the capture of only 2 Norway rats and 12 ship rats, but county rubbish tips proved to be a much more abundant source of Norway rats. Mice were captured from both sites and cultural and serological procedures revealed titres against *ballum* antigen in 10 of 79 Norway rats (13%), 4 of 16 ship rats (25%) and 10 of 67 house mice (15%). An interesting finding in this survey was a localised focus of Icterohaemorrhagiae serogroup infection in Norway rats from two county tips in Eastern Waikato, where 8 of 25 Norway rats collected (32%) had serological and/or cultural evidence of infection due to this serogroup. Five isolates were obtained and were presumed to be serovar *copenhageni*.

Icterohaemorrhagiae serogroup leptospires have been isolated from cattle in the South Auckland region (Dodd and Brackenridge,1960; Ris *et al*,1973) and, in light of the accepted worldwide role of the Norway rat as a carrier of *copenhageni*, it is probable that the Norway rat is a reservoir host for this leptospire in this region. Brockie (1977) commented on the epidemiological significance of these rodent surveys and considered that the transmission of serovars *ballum* and *copenhageni* from wild animals to humans was most likely to be effected indirectly through the agency of cattle.

Table 1.3 : Summary of methods employed and results obtained in leptospiral surveys of wildlife in New Zealand.

| Author | Animal | No. | Location | Serology | | | Culture | | | Reported serovar | Ident. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Antigens | Diag. titre | No. +ve (%) | Medium | Method | No.+ve (%) | | |
| Kirscher and Gray (1951) | Norway rat* | 53 | Dunedin Christchurch | cop,can,bat, aus.** | ? | 8 (15)*** | Gardners | cortex scraping | 2)(6) | copenageni | rabbit antisera |
| | ship rat | 47 | Auckland | | | 0 | | | 0 | | |
| Salisbury (1954) | possum | 56 | ? | pom. | ? | 0 | | | | | |
| Webster(1957) | hedgehog | 2 | Manawatu | pom. | ? | 2(100) | | | | | |
| Shortridge (1960) | "rats" | 75 | Sth.Auck. | cop. | ? | 8( 11) | | | | | |
| Blakelock & Allen (1956) | Norway rat ship rat | 62 121 | Wellington | cop,pom,can, aus,tar. | 1:10 | 0 | | | | | |
| Dodd and Brackenridge (1960) | "rats" | "few" | Sth.Auck. | ? | ? | 0 | | | | | |
| Smith(1964) | hedgehog | 104 | Hamilton, Auck.Upper Hutt,Dunedin | cop. "pool 1" | ? | 3 ( 3) | | | | | |
| Smith (1965) | hedgehog deer | 98 15 | ? | ? | ? | 2 ( 2) 0 | ? | ? | | | |
| Daniel(1966) | red deer | 102 | Kaingaroa Forest | pom,can,cop, gri,aus,hyo, and. | 1:200 | 1 (1) | | | | | |
| Daniel (1967) | wild ungulates (7 species) | 393 | various, N.Z. | as above | 1:200 | 1 (0.3) | | | | | |
| Jamieson et al,(1970) | possum | ? | ?Murchison | ? | ? | 0 | | | | | |

Table 1.3 continued.

| Author | Animal | No. | Location | Serology | | | Culture | | | Reported serovar | Ident. |
|--------|--------|-----|----------|----------|--|--|---------|--|--|-------|--------|
| | | | | Antigens | Diag. titre | No. +ve (%) | Medium | Method | No. +ve (%) | | |
| Blackmore et al(1976) | rabbit | 17 | Palmerston North | pom,cop, | 1:16 | 2 | EMJH, Fletchers and Stuarts | kidney cortex homogen- ation | 1 (6) | ballum | serology only |
| | hare | 5 | | har,bal, | | . | | | 0 | | |
| | possum | 3 | | aus,bat, | | | | | 0 | | |
| | hedgehog | 4 | | can,gri, | | 1(25) | | | 0 | | |
| | ferret | 1 | | pyr,tar | | | | | 0 | | |
| | Norway rat | 2 | | | | 2(100) | | | 1(50) | ballum | serology only |
| | ship rat | 1 | | | | | | | 0 | | |
| Buddle & Hodges(1977) | "rats" | 15 | Hutt Valley | pom,har, | | 0 | | kidney cortex homogenation | 0 | | |
| | hedgehog | 1 | | cop,bal,tar | 1:100 | 0 | Fletchers | | 0 | | |
| de Lisle, et al,(1975) | possum | 26 | Wanganui | pom,har, cop,bal. | 1:200 | 17 (65) | ? | ? | 5 (19) | hardjo | fluorescent antibody serotyping |
| Brockie, (1975) | possum | 146 | various in North Is. | har,cop,tar, bal,aut,can, pom,(and,bra) | 1:100 | 40 (27) | EMJH + antibiotics | kidney medulla maceration | 2 (1.4) | hardjo | serology only |
| Brockie & Till(1977) | hedgehog | 78 | various in North Is. | as above | 1:100 | 40 (51) | Tween 80- albumin | as above | 5 ( 6) | ballum | cross-absorption agglutination |
| Brockie,(1977) | Norway rat | 79 | various in North Is. | har,cop,bal, tar,aut,can, bra,pom | 1:100 | 11 (14) | EMJH + antibiotics | kidney medulla maceration | 13 (16) | copenhageni & ballum | |
| | ship rat | 16 | | | | 4 (25) | | | 4 (25) | ballum | |
| | house mouse | 67 | | | | 9 (13) | | | 9 (13) | ballum | serology only |

| *Common Name | Scientific Name | **Antigen abbreviations | *** Percentages in brackets |
|--------------|-----------------|------------------------|------------------------------|
| Norway rat | *Rattus norvegicus* | cop = copenhageni | |
| Ship rat | *Rattus rattus* | can = canicola | |
| Possum | *Trichosurus vulpecula* | bat = bataviae | |
| Hedgehog | *Erinaceus europaeus* | aus = australis | |
| Red deer | *Cervus elaphus* | pom = pomona | |
| Rabbit | *Oryctolagus cuniculus* | tar = tarassovi | |
| Hare | *Lepus europaeus* | and = andamana | |
| Ferret | *Mustela putoris* | gri = grippotyphosa | |
| House mouse | *Mus musculus* | pyr = pyrogenes | |
| | | bal = ballum | |
| | | har = hardjo | |
| | | bra - bratislava | |

CHAPTER II

THE NATURAL HISTORY OF THE POSSUM, *TRICHOSURUS VULPECULA*

INTRODUCTION

*Trichosurus vulpecula,* a marsupial indigenous to Australia, is a member of the Family Phalangeridae of the diprotodont sub-order of Marsupialia. This species was given the common name 'opossum' by Captain Cook who noted a superficial resemblance between this animal and the American opossum (*Didelphis marsupialis*), which is a member of the Family Didelphidae of the polyprotodont sub-order of Marsupialia (Wodzicki, 1950). The incorrect usage of the common name 'opossum' for *Trichosurus vulpecula* has persisted in Australia and New Zealand, however a change in nomenclature to the name 'possum' has been recently suggested by biologists and this is the common name that will be used in this study.

In Australia, several different subspecies of the possum have been described (Pearson, 1938; Troughton, 1946). These subspecies were differentiated on fur colour, size and behavioural characteristics, however it was noted that different strains of possums freely interbred and consequently a graded variation in fur colour and body size occurred in most localities. The most common colours were black and grey and these colours are also the most common in New Zealand (Pracy and Kean, 1969). Early liberations in this country were not differentiated as to subspecies and as all colour morphs interbred, no subspecies of *T.vulpecula* are recognised in New Zealand (Pracy and Kean, 1969).

HISTORY

The possum was introduced from Australia to New Zealand with the specific intention of establishing a fur industry. Liberations occurred throughout the latter half of the nineteenth century with a peak liberation period between 1880 - 1898 (Pracy, 1962). Many releases of progeny from the originally-introduced stock were also made and it was this artificial dispersion of New Zealand-bred progeny, both legal and illegal, that was a major contributing factor to the wide-

spread distribution of the possum throughout New Zealand (Wodzicki,1950; Pracy, 1962).

Strict protective laws and absence of natural predators aided the successful establishment of possum colonies and, by the early 1900's, thriving populations were present in many localities, both on pasture-land and in indigenous forest. As population densities increased, damage to indigenous and exotic forests, orchards, crops and gardens became evident and, from 1920 to 1946, the vigorous attempts by Acclimatisation Societies to still further disperse and establish possum populations were opposed by a growing pressure of public opinion, aware of the problems this introduced animal presented in New Zealand.

In 1947, Amendments to the Opossum Regulations (1946) removed all legislative protection of possums and, for the first time, poisoning as a means of control was legalised. A further attempt to control increasing numbers of possums was made by the introduction of a bounty of 2s.6d. per head in 1951, however this failed to achieve its objectives and empowering legislation was repealed in 1960. Under present legislation (Noxious Animals Act, 1976) control of possums is under the jurisdiction of the New Zealand Forest Service in State Forests and the regional Agricultural Pest Destruction Boards on farmland.

The possum is now present in most regions of New Zealand with the exception of some parts of Northland, Coromandel and Fiordland. Successful establishment in a wide variety of ecological niches, from exposed coastal seafaces to subalpine tussock country, has been largely due to a bountiful food supply, suitable physical habitats and an absence of natural predators. In many districts in New Zealand the possum is now present in far greater densities than in its native Australia.

BIOLOGY AND ECOLOGY

The possum is a nocturnal animal and spends the day concealed in weatherproof nests in trees, logs, holes and ground vegetation. A

nesting site is usually occupied by a single animal, although several
possums may use one nest at different times (Dunnett, 1956; Kean,1967;
Crawley, 1973), and one possum may use several nests within its
home range (Dunnett, 1956; Winter, 1963). Sharing of nests by more
than one animal at the same time can occur in areas of high
population density and limited availability of nesting sites (Kean, 1967;
Pracy and Kean, 1969).

Possums prefer a dry environment with a minimum of ground
vegetation. In forested areas, heavier population densities are
found where ungulates have opened up the forest floor, compared with
areas where dense ground cover occurs. In hilly areas, possums
prefer open ridges rather than wet gullies (Pracy and Kean, 1969).
In pastoral areas, long wet grass is avoided. The failure of
possums to become established over much of Fiordland is thought to
be due to the excessively wet climate (average annual rainfall in
excess of 500 cm).

During wet weather, possums leave their nests for only short
periods (Winter, 1963). They are rarely seen on wet nights, and
supportive evidence for their lack of movement is provided by poor
trapping, poisoning and night-shooting results during rainy weather.

The possum can utilize a wide variety of foods. Leaves form the
main part of the diet and these are supplemented seasonally by flowers
and fruit from a wide variety of trees (Wodzicki, 1950; Pracy and
Kean, 1969; Gilmore, 1965a). The possum has definite food preferences,
however if tree species of high-palatability are unavailable in a
particular ecological niche, species of lower palatability will be
eaten. On farmland, grass and clover may form a major component of
diet (Gilmore, 1965a; Quinn, 1968; Harvey, 1973). Possums have
been shown to change their feeding habits throughout the year as
changes occur in seasonally-available foods (Gilmore, 1965b;
Jolly, 1976).

Long-term trapping studies have shown the possum to be essentially
a solitary animal and there is no evidence of family or social groups
outside the mother-joey bond (Kean, 1967; Anon, 1977 ). The term

'joey' will be used throughout the study to describe pouched-young possums. Adult possums are usually sedentary and several capture-recapture studies have shown individuals to remain within the same small area for long periods (Quinn, 1968; Crawley, 1970; Crawley, 1973; Jolly, 1976).

The home range and movements of possums resident in a particular area are very variable and are a reflection of changes in behaviour associated with different population densities and changes in availability of food. The home range of forest possums has been shown to vary from 0.81 ha to 1.5 ha for males and 0.46 ha to 2.5 ha for females (Crawley, 1973). In a mixed pasture, scrub and bush environment, home ranges were found to have mean values of 0.8 ha for males and 0.3 ha for females (Jolly, 1976).

It is well established that possums will make long forays outside of their established home ranges to utilize seasonal food sources or crops. Marked animals have been shown to travel up to 1.6 km each night from a nesting site for this purpose (Tynedale-Biscoe,1955).

Although possums have been shown to have definite home ranges, territorial behaviour is either minimal or absent and fighting between males is rare (Kean, 1967; Crawley, 1973; Jolly, 1976). However observations by the author indicate that an occupied nest site will be defended against an intruder.

Ecological studies have shown that there is an extensive overlapping of home ranges of individual possums. Many possums sharing a habitat may use the same network of tracks and no area is reserved for exclusive use by an individual (Kean, 1967; Winter, 1963; Crawley, 1973; Pracy and Kean, 1969). One study reported up to 36 possums using the same communal track in one night (Anon, 1977 ), and often several possums may be found feeding in the same tree.

In contrast to adults, immature possums range over large distances before reaching sexual maturity and establishing a permanent home range (Dunnett, 1956; Gilmore, 1965b; Crawley, 1973). Immature animals may occasionally remain in the same area as their parents, especially if the population density is low (Dunnett, 1956).

The female possum has a well-defined autumn breeding season from March to May (Tynedal-Biscoe, 1955; Pracy and Kean, 1969; Crawley, 1973) and this breeding season shows little variation in length and median peak of births in different districts (Smith *et al*, 1969). As the gestation period is very short (see below),the breeding season is virtually the same as the parturient season. A second, much lesser, breeding season may occur in early spring (August and September). This second breeding season varies in intensity with locality and year, and appears to be correlated with low population densities and abundant food supplies (Wodzicki, 1950; Pracy and Kean, 1969; Kean, 1971). This lesser spring season involves both females who are breeding for a second time that year and females who have not reared a joey during the winter (Tynedal-Biscoe, 1955; Winter, 1963; Gilmore, 1969).

The female possum is monovular and polyoestrus with an average gestation period of 17.5 days (Lyne *et al*,1959; Pilton and Sharman, 1962; Kean, 1959). Parturition is followed by lactational anoestrus and the single joey, which weighs about 0.2 g when born, remains in the mother's pouch until the following spring. The age at which female possums reach sexual maturity has been reported as being from ten months to more than 24 months, together with a wide variation in the weight at which females reach sexual maturity (Table 2.1). Most workers have regarded the minimum breeding age to be one year (Tynedale-Biscoe, 1955; Gilmore, 1969; Boersma, 1974) although in forest environments females have not been found to breed until more than two years old (Crawley, 1973). Kean (1959) reported that only a small percentage of females were parous in their second year.

Most workers have considered that males also reach sexual maturity when one year of age (Table 2.1), but a detailed study by Gilmore(1969) showed that males, in general, matured approximately six months later than females and full male reproductive potential was not attained until 18 - 24 months of age. Male possums were also shown to attain sexual maturity at any time of the year. The results of Gilmore's study have been supported by work in Australia (Smith *et al*, 1969).

Weight, as with age, appears to be a poor parameter of sexual maturity in both males and females (Tynedale-Biscoe, 1955; Winter,1963;

Crawley, 1973) and therefore other biological parameters have to be used. Tynedale-Biscoe (1955) found a strong correlation between testes length in males (> 18mm) and presence of spermatozoa in the epididymis. Immature males had much shorter testis-lengths. This indicated a rapid transition between sexually immature and mature animals and this observation was also made by Gilmore (1969). The transition of immature to mature female possums is marked by the development of the pouch, and also a darkening of fur in the sternal region (Gilmore, 1969).

The obvious seasonal reproductive periodicity of the female possum is not shared by the male, which experiences constant spermatorrhoea throughout the year (Bollinger and Carrodus, 1938; Bollinger, 1946; Gilmore, 1969). Kean (1959) reported some season fluctuations in spermatorrhoea and Gilmore (1969) found that, although there was no seasonal cessation of spermatogenesis, fluctuations in the size of the prostrate occurred throughout the year and it was largest during the female breeding season. Thus the male may experience some degree of reproductive periodicity, probably directly related to the seasonal oestrus cycle of the female, but present evidence of this is inconclusive.

The reproductive efficiency of possum populations can be very high and frequently 90% of females will be carrying young by the end of the autumn breeding season (Wodzicki, 1950; Winter, 1963). Over 85% of females may conceive at the first oestrus of the season (Kean, 1959) and Crawley (1970) found that one particular female in a forest environment successfully bred each year between the ages of nine and twelve. Young females between the ages of one and two years generally have a much lower reproductive efficiency than older animals (Kean, 1959; Boersma, 1974).

A high reproductive efficiency allows possum populations to recover from control operations very rapidly. Batcheler *et al* (1967) estimated that an average kill of 67% was achieved in six poison trials in various localities. In such circumstances, with an annual reproductive potential rate of increase of 30%, remnant populations could reach pre-poison densities in as little as three years.

Pouched-young first develop fur at 70 - 80 days and do not leave the pouch for at least 112 days (Lyne *et al*,1957; Pilton and Sharman, 1962). After this time they enter and leave the pouch at will and by 150 days are riding on their mother's back (Dunnett, 1956; Pracy and Kean, 1969; Gilmore, 1969). Juvenile possums become independant at approximately 200 days (October to November). Long-term trapping studies have shown that independant juveniles generally become widely dispersed (Dunnett, 1956; Crawley, 1973), although Pracy and Kean (1969) reported that some juveniles can remain with their mother until the onset of the next breeding season.

Population structure varies considerably and reflects the history of the population (Tynedale-Biscoe, 1955). When a large proportion of juveniles occurs in a sample, it indicates that the population sampled is increasing whereas a low proportion of juveniles indicates a peak or declining population. In a study of the age structure of several populations it was found that a pasture population had a mean age of 2.8 years while two forest populations had mean ages of 3.6 and 3.9 years (Anon, 1977 ). In these studies only 10% of possums survived longer than six years and, on average, one third of the adult population died and was replaced each year. The sex ratio of possum populations has been shown to differ little from parity (Wodzicki,1950; Tynedale-Biscoe, 1955; Jolly, 1973; Crawley, 1973).

Population density is very variable. Established populations in favourable environments may reach extreme densities of up to 37/ha (Quinn, 1968). An 11 year study in a forest environment has shown fluctuations in population density of from 7 to 13 possums/ha, depending on the breeding success of the previous year and the availability of food (Anon, 1977 ). Batcheler *et al* (1967) considered that populations of around 30 possums/ha represented an average population density. A density of less than one/ha is considered light (Anon, 1977).

The damage caused by high population densities of possums is well documented. Persistent defoliation leads to death of both young and mature native forest trees and hence possums act together with goats and deer in accelerating erosion (Grant, 1956; Holloway,1959;

Table 2.1. : Weights and ages associated with the onset of sexual
maturity in the possum.

| Author | Female | | | Male | | |
|---|---|---|---|---|---|---|
| | min.age (yrs) | min.wt. (g) | usual wt. (g) | min.age (yrs) | min.wt. (g) | usual wt. (g) |
| Tynedale-Biscoe,1955 | 1 | – | – | 1 | 2300 | >2700 |
| Dunett,1956 | 1 | 1670 | >1800 | 1 | – | >1800 |
| Gilmore,1969 | <1 | – | >2000 | 1.5 | – | >2000 |
| Crawley,1973 | 2 | 1600 | >2000 | – | – | – |

Table 2.2. : Average weights of mature possums (male and female)

| Author | Weight(g) |
|---|---|
| Pracy and Kean,1969 | 3635 |
| Gilmore,1969 | 3550 |
| Crawley,1973 | 2395 |
| Boersma,1974 | 3120 |

Howard, 1964; Mead, 1976). Possums also affect birdlife by competition for food either indirectly by defoliation of trees or directly through the use or wastage of seasonal fruit and flowers (Pracy and Kean,1969). Seedlings in exotic plantations, Catchment Board plantings, orchards and crops also suffer browsing damage and in some areas possums compete directly with domestic animals for pasture (Quinn, 1968).

## ESTIMATION OF POPULATION DENSITY

The estimation of possum population densities presents many problems (Batcheler, 1973). Densities are very variable between localities and significant differences are often found between populations living in adjacent areas in the same habitat (Boersma, 1974). Absolute indices of population density have been established in some localities by the use of long-term capture-recapture studies (Winter, 1963; Anon, 1977 ) however such studies are laborious and are often a practical imposs- ibility (Caughley, 1967). Trapping to extinction has been used to calculate absolute population density (Batcheler *et al*, 1967) but this method is also laborious and contains inherent errors associated with differing susceptibilities of animals to capture and the influence of weather on trap-catch frequencies.

Spotlight counts have been used to gain rough estimates of population density and the best results are obtained during periods of good weather (Anon, 1977 ). This method has greatest application in pastoral areas and it has been calculated that 25 - 30 man-hours are required to gain a density estimate (Anon, 1977 ).

Relative indices of density can be obtained from trap-catch data, non-toxic baiting or faecal pellet counts,(Batcheler, 1973). Batcheler *et al* (1967) developed a logarithmic relationship between the frequency index of trap-catches over several nights and relative density of possum populations in different areas. This logarithmic model can also be adapted to calculate absolute density by calibration against trap-catch frequencies in a trap-to-extinction study in a known area, however the model contains several inherent sources of error. The determination of relative indices of density from trap-catch data involves the use of large numbers of traps and transects, and each estimate requires approximately 20 man-days (Anon, 1977 ).

Non-toxic baiting can be used to determine relative indices of density but this method introduces the possibility of non-random interference of baits (Bamford, 1970). This technique is carried out in forest over five fine nights and requires approximately 12 man-days per sample (Anon, 1977 ).

Faecal pellet counts appear to be the most reliable means of estimating relative indices of abundance and have been used by the New Zealand Forest Service to gauge the effectiveness of control operations in a variety of locations (Batcheler, 1971; Boersma, 1974; Spur, 1975). As with other indirect techniques of estimating population density, pellet counts are laborious in that they require large numbers of counting plots and transects. In addition, the rate of decay of pellets at the time of the year when the survey is conducted must be calculated for each locality. This method requires 30 man-days per density estimate (Anon, 1977 ). Work is presently being carried out on the calibration of the pellet-counting technique in relation to a known absolute population density in a forest area (Anon, 1977 ).

This chapter has reviewed the ecology, biology and behaviour of possums in New Zealand and provides a background against which an investigation of leptospirosis in the possum can be made. Comprehensive and accurate data on these subjects are often difficult to obtain for free-living animals compared with domestic animals and often varies according to the worker and technique employed, however it is of fundamental importance in the study of the epidemiology of disease in a free-living population.

CHAPTER III

GENERAL METHODS.

## COLLECTION OF FIELD SAMPLES

### Introduction

Three methods were used for the sampling of possum populations in the field : night shooting, trapping and poisoning.  As the study progressed, the relative merits of each sampling method under different conditions became more evident.  All methods were affected by wet weather and this often disrupted field trips.  In areas where control operations or commercial exploitation had been carried out and possum population density was low, many more days in the field were necessary as compared with those required for areas where the population density was high.

Night-shooting operations were carried out with the indispensable help  of Agricultural Pest Destruction Board (APDB) employees, otherwise, possum populations were sampled by the author by trapping or poisoning.

### Night-Shooting

Night-shooting proved to be a good method for sampling possum populations when weather conditions were suitable.  The best results were obtained on the first fine night following rain.  Wet or excessively windy nights were unsuccessful as the possums tended to remain in shelter under these conditions.

Four-wheel drive vehicles owned by the APDB were used for night-shooting operations.  Possums were head-shot with 0.22 calibre rifles equipped with telescopic sights.  Hand-held and helmet-mounted spotlights were used to locate possums and most animals were shot from trees.  Possums on the ground usually ran to the nearest tree when disturbed.

It was found that head-shot possums bled profusely for a period of up to 30 seconds following shooting and therefore rapid recovery of the carcass was required for the collection of blood.  Accurate shooting was essential as it was difficult to get blood samples from body-shot animals.

Head-shooting was also necessary for effective post-mortem and microbiological procedures to be carried out later.

Blood was collected in rubber-stoppered tubes [a]. Each possum was ear-tagged with a tag from a consecutively-numbered series and this number was marked on the blood collection tube. Bloods were held at ambient temperature until the following day.

Night-shooting had several advantages over other methods and a subjective assessment of the relative efficiency of this method compared with trapping and poisoning is given in Table 3.1. It was the most efficient method in summer in areas where possums were plentiful and it allowed simultaneous subjective estimations of population density by spotlight counts to be made. Repeated samples of possums could be taken from the same environment without affecting population density, as operations could be conducted over a large area. Working from a vehicle also meant that carcass recovery for post-mortem and microbiological procedures was easily achieved.

A severe disadvantage of night-shooting was its restriction to use in fine summer weather. Very few possums were seen on rainy nights and, during winter, farm tracks became impassable to the four-wheel drive vehicles. Night-shooting was also limited to areas of APDB activity and also to areas that had vehicular access.

Success of night-shooting operations varied considerably. The highest tally in one night for two shooters and one collector (21 man-hours) was 82 possums and 73 blood samples. On several occasions when conditions were unfavourable, less than five possums were shot. Most night-shooting was done by using one shooter and one collector.

## Trapping

Trapping with a spring-jaw trap is a very efficient, though time consuming, method for obtaining possums and is widely used by commercial operators. It provides a good means for sampling low-density populations and, of the three sampling methods, is the least affected by wet weather.

[a] Vacutainer, Becton-Dickinson and Company, U.S.A.

Although efficient, this method of trapping is inhumane and therefore use in the present study was restricted to those situations where other methods could not be applied. These included the sampling of a population on Otawhao farm and the gathering of possums when night-shooting was not possible. Traps were also used in some forest areas.

Traps were set on possum runs and against bases of trees and were baited with a mixture of flour and clove oil. Trapped animals were stunned by a sharp blow on the head and bled from the jugular vein into a collecting tube. Ear tags were used as for shot animals.

Advantages of trapping included the effective capture of possums under a wide range of conditions, the possibility of use in wet weather and the provision of trap-catch frequency data for estimates of relative population density. Trapping was also an efficient means of sampling low density populations.

In addition to the inhumane nature of the method, a major dis-advantage was the time involved in the setting up and servicing of traplines, especially in areas where access was difficult.

## Poisoning

Sodium cyanide poison, applied in paste form [b], has been found to be an effective means of killing possums and is used in preference to traps by many commercial possum hunters because of its ease of application. It is also used by control agencies in ground control operations in peripheral areas following aerial poisoning programmes. The poison is most effective for high and moderate density possum populations and a single operator can cover a large area and lay many hundreds of baits, however "bait-shyness" may occur in populations that have been previously poisoned. This can severely limit poisoning effectiveness (N.Lee, pers.comm.)

Cyanide poison was applied under the Agricultural Chemicals (Vert-ebrate Pest Control) Regulations (1976). Flour and clove oil lure

[b] Wanganui Poison Factory, P O Box 333, Wanganui, New Zealand.

Plate 3.1: Steel trap of the type used for the
sampling of some possum populations.

Plate 3.2: Cyanide paste bait eaten by a possum.
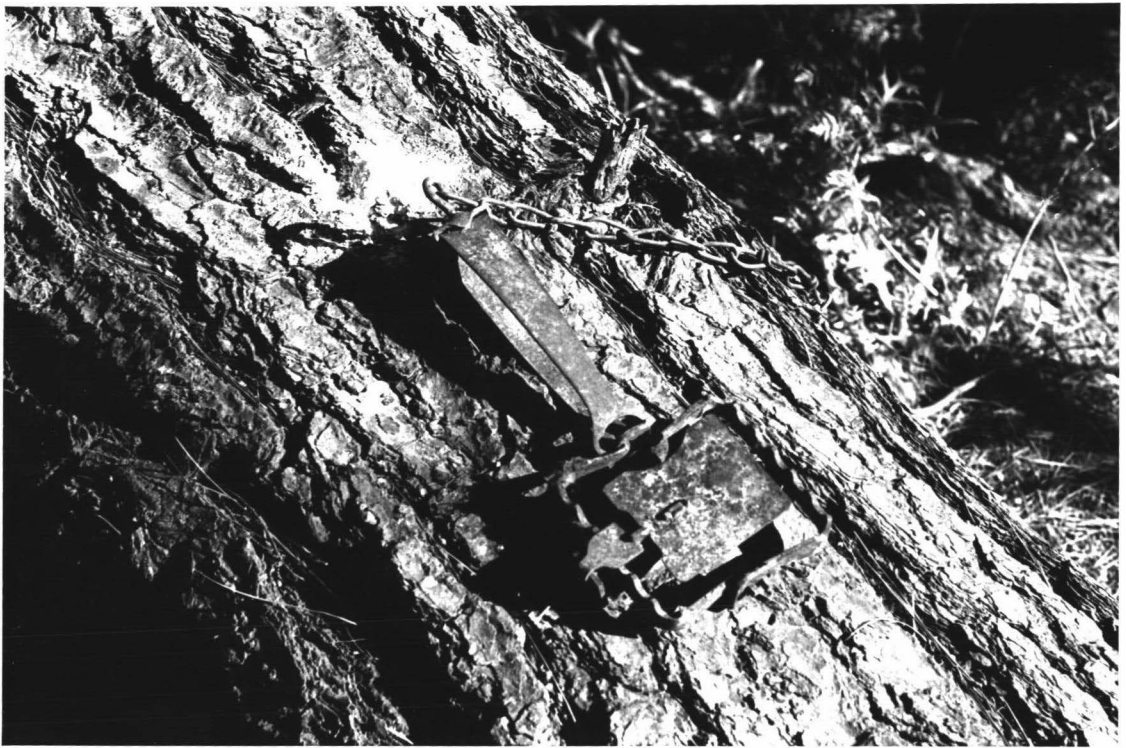
was used. The poison paste was applied to the base of trees and logs and stones on possum runs and a small amount of lure was sprinkled over the bait.

In the present study, cyanide poison was used extensively in forest areas where trapping was impractical. It was also used in some pastoral areas when night-shooting was not possible.

Carcasses were recovered as early as possible the morning following baiting. These were ear-tagged and blood samples were obtained by sectioning the *venous arcus hyoideus*, a large vein lying ventral to the thyroid gland and joining the left and right jugular vein. The possum was held upside down and up to five mls of blood could be obtained by massaging the jugular vein towards the head. This was collected in a blood-collecting tube held under the throat.

The sera from cyanide-poisoned possums was more haemolysed than sera from trapped or shot possums. Comparative studies showed that there was no statistical difference between the leptospiral titres of sera obtained from poisoned animals when compared with sera obtained by other methods (see Chapter IV). This method of serum collection may prove useful in other disease surveillance programmes.

## Capture of Live Animals

Laboratory studies on the pathogenesis of leptospiral infection in possums required the capture of live adult and juvenile possums. Walk-in cages were used where access to trapping sites was reasonable but these cages were too bulky and inefficient for general use. The low trapping efficiency which was experienced was due to possums either being trap-shy or else springing the trap without being caught.

Juvenile possums (and some adults) were caught alive by hand. This method required a pastoral environment, preferably hilly and with a limited amount of cover or trees. Possums out in the open on the ground were located with spotlights and run down and caught by the tail before they could reach cover or climb a tree. They were suspended by the tail and immediately transferred to a draw-neck bag to avoid danger

Table 3.1 : Subjective assessment of relative efficiency of sampling
methods for investigation of leptospirosis in moderate-
density possum populations.

| | Method | | |
|---|---|---|---|
| Condition | Night-Shooting | Trap | Cyanide Poison |
| wet weather | + | +++ | ++ |
| summer | +++ | + | ++ |
| winter | + | +++ | ++ |
| pasture | +++ | + | ++ |
| forest | + | ++ | +++ |
| repeat sampling | +++ | ++ | + |
| population estimation | +++ | ++ | + |
| least man-hours | +++ | + | ++ |

to the handler by clawing and biting. This technique was especially
effective for catching juveniles that were non-dependant on, but
still in association with, their mother. Juveniles were only rarely
caught in walk-in traps.

A small number of juveniles used in laboratory studies were those
being carried on the back when the mother was shot. This source of
experimental animals was only available during a short period of the
year however.

## Processing of Possums

On the return to the laboratory or field station, possums were
weighed, sexed and classified as to age-group. Coat colour was also
noted. Three age-groups were used in this study : juveniles,
transitional-stage adults and mature adults. Juvenile males (JM)
were classified as those animals that had a testes length of less
than or equal to 18mm (Tynedale-Biscoe,1955). Calliper measurements
were made through the scrotal sac. Juvenile females (JF) were
classified as those animals that had no pouch development (Gilmore,1969).
A secondary characteristic of sexually-immature males and females was
the lack of fur discolouration over the sternum (Gilmore,1969).

Transitional-stage adult males (TM) and females (TF) were classified
as those animals that had achieved sexual maturity but lacked the body
weight and conformation of fully mature adults. Transitional-stage
adults generally consisted of those possums in the population between
one and two years of age. In forested areas, possums in this group
may have been up to three years of age.

The transitional-stage adult group in a population would contain
a proportion of the animals classified by Jolly (1973) as the sub-adult
group. This author used a testes length of less than 21mm in males
and independance from the mother to define the upper and lower limits
of animals within this sub-adult group. In this scheme of class-
ification,sub-adult and adult females could not be reliably distinguished.

Mature adult males (LM) and females(LF) were classified as those
animals weighing more than 2,400 grams and having a fully-mature body

and head conformation. Although conformation was a subjective parameter of age-group in this classification system, there was an obvious difference between fully mature adults and transitional-stage adults. Possums in the mature adult group were considered to be a greater than two years of age.

The culture method for isolation of leptospires from possum kidneys is described later in this chapter.


LABORATORY METHODS

The laboratory diagnosis of leptospirosis depends on three techniques : the direct microscopic detection of leptospires in host tissues or fluids, the demonstration of leptospiral antibodies in serum and the isolation of the infecting organism by animal inoculation or direct culture in artificial medium. This section describes these techniques as applied in the present study.

### 1. Direct microscopic detection of leptospires in tissues and body fluids.

Leptospires can be detected in blood, urine and tissue homogenates by several techniques, the most common of which are dark-field microscopy, fluorescent antibody techniques and silver-impregnation of formalin-fixed tissues or smears. Other techniques that have been described include tissue imprints and direct staining (Sturdza and Safiresco, 1964) and fluorescent antibody tests on scrapings from paraffin-embedded tissue blocks (Sulzer and Jones, 1974). These latter techniques have yet to be fully evaluated.

In this study, dark-field microscopy was used where applicable for the examination of tissue homogenates and body fluids. This was carried out on a Leitz[c] dark-field microscope at 156 X magnification.

[c] Leitz Wetzlar, Ernest Leitz G.M.B.H. Wetzlar, Germany.

Tissue homogenates were examined by transfering a small drop with a platinum loop to a glass slide and distributing the drop in a thin layer under a cover slip. Tissue homogenates from the 1:50 and 1:500 dilution (see culture methods) were examined by this method.

Blood for dark-field examination was collected in heparinised tubes and centrifuged at 500G for 5 minutes to remove cellular elements from the plasma, a drop of which was then examined. A two-speed centrifugation technique described by Wolff (1954) was also used in the early stages of the study. This involved a second, high-speed centrifugation to sediment leptospires suspended in plasma. The identification of leptospires in this sediment was very difficult because of the presence of protoplasmic extrusions from blood cells and other artifacts, and the use of this technique was discontinued.

Urine samples were taken mid-stream from experimental animals and by bladder puncture from animals at necropsy. Urine was centrifuged at 500G for five minutes to remove cellular elements and crystalline debris and a loop of supernatant was examined by dark-field microscopy for leptospires. The supernatant was then centrifuged at 14,000G for 30 minutes and a portion of the sedimented pellet was resuspended in a drop of saline on a glass slide and examined for leptospires.

Although leptospires have characteristic morphology and motility, cellular extrusions and miscellaneous debris can easily be mistaken for non-motile organisms in wet preparations. In addition, more than 10,000 leptospires per ml must be present in the test material to detect one leptospire per high-power field (Turner, 1967). Therefore, failure to detect leptospires by this method does not rule out their presence. For these reasons, dark-field examinations are unreliable as a diagnostic procedure (Wolff, 1954; Alston and Broom, 1958; Turner, 1967; Sulzer and Jones, 1974) and in this study they were only used as an adjunct to serology and culture. A positive identification of leptospires in a sample was only made if two or more motile organisms were seen in the sample.

A modified Warthin-Starry technique was used for the demonstration of leptospires in formalin-fixed paraffin tissue sections (Young, 1969). Silver-impregnation techniques for the demonstration of leptospires have similar limitations as dark-field microscopy and therefore this

technique was not routinely used as a diagnostic procedure.

## 2.   Serology

### Introduction

Serological procedures can be used to detect leptospiral antibodies
in infected animals and also to identify leptospires isolated in
artificial media.  The microscopic agglutination test (MAT) is the most
common method used (Alston and Broom, 1958; Galton *et al*, 1962; Cole
*et al*, 1973).  This test can be used with both live and formalinised
antigens and has a high sensitivity and specificity (Turner, 1968).  The
MAT has recently been modified for use in microtitre plates so as to
increase efficiency and decrease the amount of antigen necessary for
the test (Galton *et al*, 1965; Cole *et al*, 1973; Sulzer and Jones, 1974).

A macroscopic agglutination test may also be used to detect
leptospiral antibodies in sera.  Although quick and easy to perform,
this test is poorly serovar-specific and is usually used only where
the preliminary screening of a large number of sera is required (Wolff
1954; Turner, 1967).

Other techniques used in the serological diagnosis of leptospirosis
include complement fixation (CF), sensitised erythrocyte-lysis (SEL),
haemagglutination (HA), direct and indirect fluorescent antibody (FA)
and *patoc* agglutination.  These tests are generally only genus-specific
and are often difficult to standardise and perform (Sulzer and Jones,
1974).  They have more application for use on human sera than animal
sera because non-specific reactions and cross-reactions limit effectiveness
in the latter group (Palit and Sharma, 1971; Tan and Welch, 1974; Pinto
*et al*, 1974).  Sulzer *et al* (1975) developed a genus-specific indirect
HA test for use on human sera that had 92% sensitivity and 97%
specificity when compared with the MAT.  This test has the advantage
of being relatively simple to perform but it has not been evaluated for
use in animal sera.

## (i) Microscopic Agglutination Test

Serological examinations in this study were performed using the MAT. The method used was a modification of the micro-technique described by Galton *et al* (1965) and Cole *et al* (1973). Leptospiral isolates were also identified using this method.

Sera to be tested were dispensed in 50 µl volumes into individual wells in a microtitre plate [d]. Each plate could hold a maximum of 88 sera and eight saline or standard antiserum controls. A pipetter machine [d] was used to add 100 µl of physiological saline to each well to produce a serum dilution of 1:3. This plate was referred to as the serum reference plate (SRP) and could be used immediately or sealed with tape [d] and stored at $-20^{\circ}$C for future use.

A series of microtitre plates were prepared by dispensing 25 µl of saline into each well, using the pipetter. The SRP was then placed on the tray of a dilutor machine [d] and 25 µl of each of the 12 sera in the first row picked up with the dilutor heads. These sera were mixed with 25 µl of saline in the first row of a transfer microtitre plate (TP) and then transferred with the dilutor heads to another microtitre plate containing saline. This produced serum dilutions of 1:12 in the first row of wells, and sequential dilutions down the plate produced doubling dilutions of each serum of from 1:12 to 1:1,536. Successive rows of sera from the SRP were diluted in a series of microtitre plates in the same manner.

The choice of possible serum dilutions was limited due to the 25µl capacity of the dilutor heads and the limited capacity of the wells in the microtitre plates. The dilution system chosen was the closest approximation to the optimum dilution system of 1:25, 1:50, 1:100 etc. that could be conveniently attained.

Live antigen was dispensed by hand using a modified Cornwall [e] syringe to which a minipipetter dispensing head[d] had been attached. The Cornwall syringe was modified so that a 15ml narrow-necked bottle of antigen could be attached by a screw thread to the base of the

[d]Dynatech Laboratories Inc., Alexandria,Virginia,U.S.A.
[e]Becton-Dickinson and Company,U.S.A.

barrel (Plate 3.3.). The antigen could thus be dispensed without danger of spillage.

Antigen was added in 25μl aliquots to each well in the microtitre plates to produce final doubling dilutions of each serum of from 1:24 to 1:3,072. The test plates were then covered with cover plates and incubated for 90 minutes at $37^{\circ}C$.

Following incubation, sera were transferred to glass slides using a special instrument (Ryan, 1978). This apparatus consisted of eight stainless steel rods held in a block in such a way that they would fit into a row of eight wells in a microtitre plate. This enabled each of the eight rods to pick up a drop of sera and transfer it to a slide for examination. In this way the complete doubling dilution series of each serum sample could be transferred to a slide at the same time.

The titre of an agglutination reaction was taken as the final dilution of serum at which 50% or more of the leptospires were agglutinated or lysed, as observed by darkfield microscopy (Anon, 1967).

(ii) Antigens

Leptospiral serovars used for antigen production were maintained in liquid EMJH culture medium (see cultural methods) and subcultured every seven days. Approximately 10% of an actively-growing culture was used as an inoculum for each subculture. Cultures were held at $30^{\circ}C$ and examined by dark-field microscopy for density, homogeneity and lack of contamination before use. Antigens of a density lower than $1 \times 10^8$ organisms/ml were not used, as increased titres have been shown to occur with low-density antigens (Borg-Petersen and Fagroeus, 1949; Anon, 1967). Optimum density for antigens was $2 \times 10^8$ organisms/ml. Very dense cultures were diluted with liquid media to this density before use. Only serovars of standard strains were used as antigens, because of the variability in titre that can occur when a serum is tested with different strains of the same serovar (Borg-Petersen and Fagroeus, 1949; Turner, 1968).

The sensitivity of each batch of antigen was checked against the homologous standard antiserum at the time of use. Antigens that gave
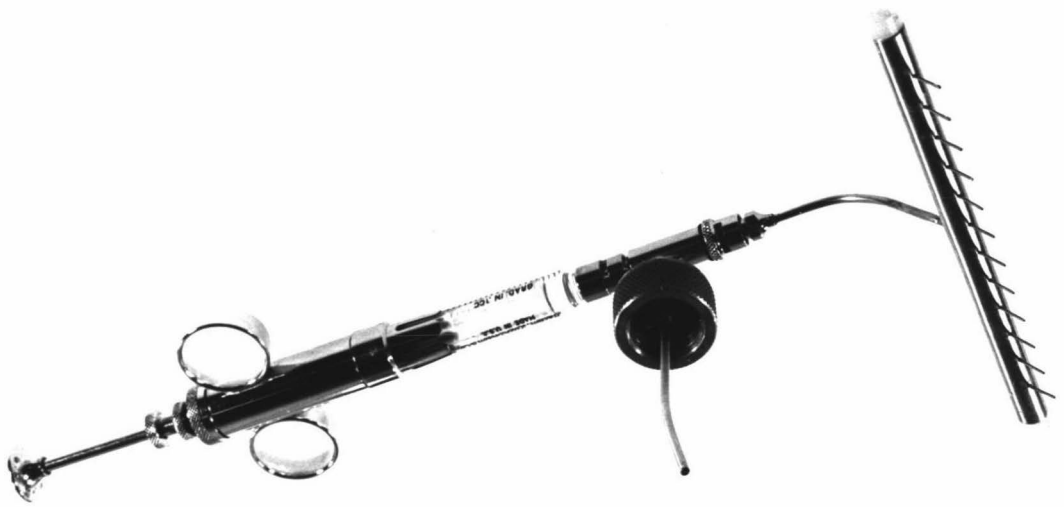
Plate 3.3. : Modified Cornwall syringe for
dispensing antigen.

a titre greater than a two-fold dilution above or below the known titre of the antiserum were discarded.

## (iii)    Identification of Leptospiral Isolates

Leptospiral isolates were grown in liquid media to a minimum density of $1 \times 10^8$ organisms/ml and then used as antigens in the MAT for serogroup identification.  All isolates were tested against a battery of 12 standard antisera produced in rabbits against serovars *hardjo* (strain hardjoprajitna), *pomona* (pomona), *ballum* (Mus 127), *copenhageni* (M20), *tarassovi* (Mitis Johnson), *bataviae* (Swart), *canicola* (Hond Utrecht), *pyrogenes* (Salinem), *grippotyphosa* (Moskva V), *autumnalis* (Akai), *australis* (Ballico) and *biflexa*[f] (CDC).  The initial antiserum dilution used in the MAT was 1:96.  Serogroup classification of isolates was determined by the pattern of cross-agglutination titres produced against these standard antisera.

When definitive serovar identification was required, isolates and specific antisera were sent to the Leptospirosis Laboratory, Centre for Disease Control, Atlanta, Ga., U.S.A. for cross-agglutination absorption studies using the method described by Kmety *et al* (1970).  The production of specific antisera in rabbits against isolates sent for serovar identification is described in Appendix I.

A minimum serum dilution of 1:24 was used in serological surveys, however in some circumstances a lower initial serum dilution of 1:12 was required.  This was achieved by discarding the use of the transfer plate.  If an initial serum dilution of 1:6 was required, the dilution of serum in the SRP was reduced from 1:3 to 2:3.

Sera with titres of greater than 1:3,072 were retested in a doubling dilution series up to a serum dilution of 1:98,304.

## 3.    Culture methods

Introduction
    The identity of an infecting leptospiral serovar can only be

[f] serovar supplied by CDC, Atlanta, U.S.A., Strain unknown.

determined by isolation of the organism from the host and typing
according to standard techniques. Hence, an efficient culture method
for the recovery of leptospires is a very important part of any
epidemiological investigation.

In the past, media for the cultivation of leptospires were enriched
with animal serum, usually that of rabbits (Vervoot, 1922; Fletcher,1928;
Stuart, 1946; Chang,1947). Such media supported the growth of most
leptospiral serovars however a variety of problems, particularly
associated with the rabbit serum supplement, limited their effectiveness.

Investigations of the specific nutritional requirements of leptospires
showed that growth could be sustained in a serum-free media that contained
albumin, long-chain fatty acids, vitamins $B_1$ and $B_{12}$, essential mineral
salts and water (Schneiderman $et$ $al$, 1953; Johnson and Gary,1962; 1963a;
1963b). The first media using bovine serum albumin (BSA) and Tween 80
as a source of long-chain fatty acids was described by Ellinghausen
and McCullough (1965). A modification of this BSA-Tween 80 medium
(Johnson and Harris,1967) is now widely used as the medium of choice
for the cultivation of leptospires.

It has been well established that BSA-Tween 80 medium promotes
good growth of leptospires and permits the isolation of serovars
that will not grow in media enriched with serum. The medium has a
long shelf life and the antigenicity and immunogenicity of leptospires
cultured in BSA-Tween 80 do not differ from those grown in media
containing serum (Auran $et$ $al$,1972).

(i) Media

The culture medium used in this study for leptospiral isolation
and maintenance was EMJH[g] , a commercially prepared BSA-Tween 80 medium
(See Appendix II). The addition of agar enables media to support
more rapid, dense and sustained growth of leptospires compared with
liquid media (Turner,1970; Tripathy and Hanson,1974). Semisolid EMJH
(0.15% agar w/v) was therefore used for isolation of organisms and
maintenance of stock cultures. Liquid media was used for the main-

[g]Difco, Detroit,Michigan, U.S.A.

enance and growth of antigens and for the serotyping of isolates.
Leptospires isolated in semisolid medium required several passages in
liquid media to remove all traces of agar before being serotyped. This
was necessary because small clumps of agar interfere with the inter-
pretation of serological tests.

Media was dispensed in five ml aliquots in 15 ml narrow-neck
bottles and held at $37^{o}$C for three days and $30^{o}$C for three days to
detect any bacterial contamination. Liquid medium for antigen
production was dispensed in larger volumes as required. Each batch
of medium was checked for the ability to grow leptospires by the use
of small inocula of a recent isolate of serovar *hardjo*($10^{1}$, $10^{2}$,
$10^{3}$, $10^{4}$ and $10^{5}$ organisms), a strain with fastidious growth requirements.

Leptospires are very susceptible to competition from other micro-
organisms and the incorporation of 5-Fluorouracil[h] (5FU) in semisolid
media has been shown to selectively inhibit contaminating bacteria
(Johnson and Rogers,1964). For each culture sample in this study, a
series of inoculations were made in media containing 200 μg 5FU/ml
(see Appendix II) and a parallel series were made in media without
this selective agent. Both types of media were used in parallel as
it has been reported that 5FU may inhibit some leptospiral strains
in primary cultures (Turner,1970). A comparison of the effectiveness
of these culture regimes is given in Chapter V.

(ii) Culture from body tissues and fluids

The choice of suitable material for culture depends on the likely
stage of infection of the host under examination (Turner,1970). If
the animal is in the leptospiraemic stage of infection (usually days
one to eight), blood and highly vascular organs such as liver and
kidneys are the materials of choice. Isolations may also be from
cerebro-spinal fluid. During the leptospiruric phase of infection
(usually from day seven onwards), the renal cortex and urine are the
most suitable materials for culture. Some workers have recovered
leptospires from the brain of infected animals (Sleight *et al*, 1960),
however the reliability of culture of this organ from infected animals

[h]Sigma Chemical Co., St.Louis, Missouri,U.S.A.

is not known.  Wolff (1965) reported negative results from brain cultures
of hedgehogs that had a high prevalence of kidney infection.

The majority of a population of wild animals in which endemic
leptospirosis occurs are unlikely to be in the leptospiraemic phase
of infection when sampled and therefore kidneys and urine are the most
suitable materials for culture.  These were the materials cultured in
field surveys in this study.

Kidneys were removed from possums as soon as possible after death.
Prior to necropsy the animals fur was swabbed with a solution of 2% Medol[i].
Instruments were dipped in 70% alcohol and sterilized by flaming, and
the abdominal muscles were exposed by cutting down the midline and
reflecting back the skin.  A new set of instruments were used to open
the abdominal cavity and asepticaliy remove the kidneys, which were
placed in sterile,disposable petri dishes.  One kidney was retained
for histological purposes and the other was used for culture.  Urine
from the bladder, if present, was aspirated with a two ml,sterile
disposable syringe fitted with a one inch x 22 gauge needle.  If a
dark-field microscope was available, urine was examined immediately
for the presence of leptospires.

Homogenising of kidneys was carried out in a Coleworth Stomacher
400[j].  Each kidney was aseptically transferred to a $\gamma$ ray-sterilised
plastic bag containing 50 mls of Stuarts base medium (SBM) (Stuart,1946)
and homogenised for two minutes.  This provided an initial kidney
homogenate of approximately 20% w/v.  The bag was removed from the
Stomacher and one ml of homogenate inoculated into nine mls of SBM.
Two serial ten-fold dilutions in SBM were made from this initial dilution
to make final kidney tissue dilutions of 1:50, 1:500 and 1:5000.  These
were designated K-1, K-2 and K-3.  Approximately 0.25 ml of each
dilution was pipetted into a bottle of semisolid media and a bottle of
semisolid media containing 200 $\mu$g 5FU/ml.

The Stomacher could not be used for small amounts of tissue and

[i]William Pearson Ltd., Auckland, New Zealand
[j]A.J.Seward and Co.Ltd., London,England.

kidneys weighing less than two grams, such as those from pouched-young joeys, were homogenised by forcing the kidney through a five ml, sterile disposable syringe fitted with a 0.5 inch, 14-gauge needle. The homogenate was inoculated directly into nine mls of SBM and diluted as already described. Hamster kidneys and livers, and a small number of possum brain samples, were homogenised in the same way.

Experimentally-infected possums and hamsters were bled by heart puncture and one or two drops of blood were inoculated directly into each of two bottles of semisolid media and two bottles of media containing 5FU (anaesthesia and handling procedures for experimental possums are described in Chapter VII). Minimal inocula of blood were used as large volumes fo blood inhibit the growth of leptospires in culture media (Sulzer and Jones, 1974). Media were inoculated immediately after the blood sample was taken, hence anticoagulants were not necessary in blood-collecting tubes.

Mid-stream urine samples from experimentally infected possums were collected in sterile, 25 ml bottles. These were diluted 1:50 and 1:500 in SBM and two drops from each of the neat and diluted urine samples were inoculated directly into semisolid media containing 5FU. A series of media with 5FU were also inoculated. As with blood, larger inocula were avoided as they have been reported to inhibit leptospiral growth (Galton *et al*, 1962; Turner, 1970). The same procedure was used for urine samples collected at necropsy.

Voided urine was generally grossly contaminated with micro-organisms and therefore filtration and hamster inoculation techniques were employed, in addition to 5FU, in an attempt to overcome the problem of contamination of cultures. Five mls of the urine to be filtered were centrifuged at 500G for five minutes and supernatant was drawn into a five ml, sterile disposable syringe. A Swinnex[k] filter, loaded with a 0.45 μm pore size membrane[k] and prefilter, was attached to the syringe. Approximately one ml of urine was expressed through the filter and then two or three drops of filtered urine were inoculated directly into each of two bottles containing semisolid media and two bottles containing semi-solid media plus 200 μg 5FU/ml.

[k]Millipore Corp., Bedford, Massachusetts, U.S.A.

(iii) Hamster inoculation

The use of laboratory animals provides no greater chance of leptospiral isolations from material obtained aseptically than direct culture techniques, however they are especially useful for the isolation of leptospires from material containing contaminating micro-organisms (Turner,1970).

Hamsters were used extensively in this study for isolation of leptospires from contaminated tissues and fluids and also for environmental sampling of surface water and soil. Weanling hamsters (20 - 30g) were inoculated by the intra-peritoneal route with one ml of contaminated material and examined daily for clinical symptoms. The liver and kidneys of moribund or dead hamsters were cultured as previously described. Hamsters surviving 21 days after inoculation were anaesthetised with ether and bled by cardiac puncture for serological examination. They were then killed by cervical dislocation and the kidneys cultured.

(iv) Maintenance of cultures

All primary cultures in semi-solid medium were held at $30^{o}C$ for three months. They were examined for growth by dark-field microscopy at two-weekly intervals for two months and then again at three months before being discarded if still negative. As soon as growth was detected in primary cultures, a 10% inoculum was transferred to liquid medium for growing up and serotyping. Positive cultures were also subcultured into fresh semi-solid medium for maintenance.

Stock antigens were maintained in duplicate in liquid media and transferred every seven days. Periodic checks for bacterial contamination were made by plating out on blood agar which was incubated aerobically for five days. Stock cultures were maintained in semi-solid media and subcultured every four months. A duplicate series of stock cultures was held at room temperature in the dark and subcultured every six months.

If cultures became contaminated, decontamination was attempted by filtration (see above), hamster inoculation or subculturing into media containing 400 μg 5FU/ml.

This chapter has described the general materials and methods used throughout this study.  Some changes were made to the general methods as the study progressed, and these and details of specific techniques are described in subsequent chapters.

CHAPTER IV

SEROLOGICAL SURVEY OF PASTORAL POSSUM POPULATIONS

INTRODUCTION

Leptospiral infection in possums was first reported by Brockie (1975) and de Lisle *et al* (1975). Previous workers (Salisbury, 1954; Jamieson *et al*, 1970) had made brief mention of limited numbers of possum sera being examined, with negative results. Salisbury (1954) tested 56 sera against only *pomona* antigen and Jamieson *et al* (1970) provided no data on number of sera tested or antigens used. Seven sera from possums from Tasmania, Australia were also found negative against seven antigens (Munday, 1972). The identity of the antigens used was not specified.

Brockie (1976) collected a total of 146 possums from a variety of locations and found 38 (26%) had titres against *hardjo* antigen at an initial serum dilution of 1:100. Two isolates were recovered from culture of kidneys (1.5%) and these were serotyped by cross-agglutination and reported as being serovar *hardjo*.

De Lisle *et al* (1975) found 17 of 26 possums (65%) seropositive to *hardjo* antigen at an initial serum dilution of 1:200. Five isolates were obtained from the 26 possums (19%) and these were typed by fluorescent-antibody serotyping as also being serovar *hardjo*.

These initial reports of *hardjo* infection in possums were of considerable interest. *Hardjo* has not been isolated from any free-living species in other countries and therefore the presence of a wildlife reservoir in New Zealand would be unique. If such a reservoir was present, it may have also been important in the epidemiology of *hardjo* infection in bovines and humans in New Zealand.

At the beginning of the present study, a serological survey was undertaken in the southern half of the North Island of New Zealand to determine the prevalence of leptospiral titres in possum populations inhabiting pastoral areas. Samples were taken from a variety of locations and types of farms. The results of this serological survey are reported in this chapter.

MATERIALS AND METHODS

Possums were collected mainly by night-shooting with APDB personnel. In areas where night-shooting was not possible, populations were sampled by poisoning or trapping. The geographical locations from which samples were obtained in this study and from which samples were obtained by Brockie (1975) and de Lisle *et al* (1975) are shown in Figure 4.1.

1.    Linton.  July 1975.

Possums were collected on two adjacent, intensive sheep and beef farming properties.  The population density was low due to the limited availability of shelter and only 14 possums were obtained.  The sample was collected by trapping.

2.    Greenmeadows Farm, Ohau.  July 1975.

This 90 ha dairy farm was located in the centre of a dairying district and was stocked exclusively with dairy cows.  Flat, fully-developed farmland provided little shelter for possums, apart from isolated trees and some boxthorn shelter belts.  Population density was very low and only 12 possums were collected by trapping and shooting over two nights.

3.    White Rock Station, South Wairarapa.  August 1975.

This 6000 ha beef and sheep farm consisted of rolling to steep hill country with an abundance of scrub in gullies and on some undeveloped parts of the station.  Population densities were high and possums were observed at night to range over wide areas of pasture.  A total of 110 possums was obtained by poisoning and trapping over four nights.

4.    Ngamu Station, Masterton.  August 1975.

Ngamu Station is a sheep farm bordering the Ngamu State Forest east of Masterton.  The pastoral possum population was thought to be continually reinforced by possums from the neighbouring exotic forest and population density was moderate.  Shelter was provided by small pockets of remnant native forest and bands of willows along creek beds.  Forty possums were taken by trapping for two nights.

5.   Woodville.  August 1975.

Possums were collected from several farms east of Woodville.  Land-
use was mixed, with darying on the flats and sheep and beef farming on
the easy hill country.  Pockets of scrub and regenerating native bush,
shelter belts and willows provided good nesting sites and possums were
present in high density.  A total of 95 possums were obtained by night-
shooting over two nights.

6.   Dannevirke.  September 1975.

The sampling locality six miles southeast of Dannevirke was
similar to that described for Woodville.  Population density was
moderate.  Night-shooting over two nights resulted in a sample of 48
possums being taken.

7.   Ormondville, Sample 1.  September 1975.

Possums were obtained from several sheep and beef farms near Te Uri,
Ormondville.  Shelter was provided by small pine plantations, shelter
belts, pockets of scrub and willows.  Spotlighting revealed many possums
in close proximity to domestic stock on pasture.  Population density
was moderate and 91 possums were obtained by night-shooting over two
nights.

8.   Craigs Farm, Ormondville, Sample 2.  September 1975.

A second sample from Ormondville was taken from a farm near the
location from which the first sample was taken.  Possums were found in
the willows along creek beds where they were feeding on new willow
shoots.  Population density was moderate.  Forty-six possums were
taken over two nights by night-shooting.

9.   Tukituki.  October  1975.

A sheep and beef farming area near Tukituki was sampled over one
fine and one wet night.  Population density was very low and night-
shooting produced a sample of only seven possums.

10.  Porangahau.  October 1975.

This coastal farming district consisted of rolling to steep hill country with extensive areas of scrub and gorse separating areas of farmland.  Possums had adequate shelter and fed on pasture at night. Population density was moderate.  Two nights shooting produced a sample of 54 possums.

11.  Pahiatua.  October 1975.

Possums were shot on a hill country sheep and beef farm south-east of Pahiatua.  Scrub and regenerating native bush in gullies provided cover for a population of moderate density.  A sample of 66 possums was taken over two nights.

12.  Paekakariki.  November 1975.

The farm on which this sample was taken ran stud Charolais cattle and sheep on coastal sand and peat country.  Haybarns, small patches of lupin and a few shelter belts provided limited shelter for possums, which were present in low numbers.  A sample of 17 possums was taken over two nights by trapping and poisoning.

All sera were tested by the MAT with serovars *hardjo, ballum, copenhageni, pomona* and *tarassovi* as antigens.  These were the five leptospiral serovars that had been isolated in New Zealand at that time.  In addition, 120 randomly-selected sera were tested against serovars *canicola, bataviae, grippotyphosa, pyrogenes, autumnalis, australis* and *biflexa*.

The precision of the MAT system used in our laboratory was determined by the repeat testing of 100 randomly-selected sera, six months after the initial test.  The *hardjo* antigen used was tested against a standard antiserum and the repeat titre was within one dilution of that given by the antigen used in the initial test.

All titres recorded to antigens other than *hardjo* were confirmed by repeat testing of the sera involved.  Analysis of results was achieved by transforming titres into coded titres units (Appendix III).

Figure 4.1. : Locations from which pastoral
possum samples were taken.

* Locations sampled by
  Brockie (1975.)
** Location sampled by
  de Lisle *et al* (1975).

* Helensville

o *Putaruru

* South Taranaki

**Wanganui

Porangahau

Tukituki

Ormondville

Dannevirke

Palmerston North

Woodville

*South Manawatu — Linton

Ohau

Pahiatua

Paekakariki

Masterton

* Wellington

White Rock
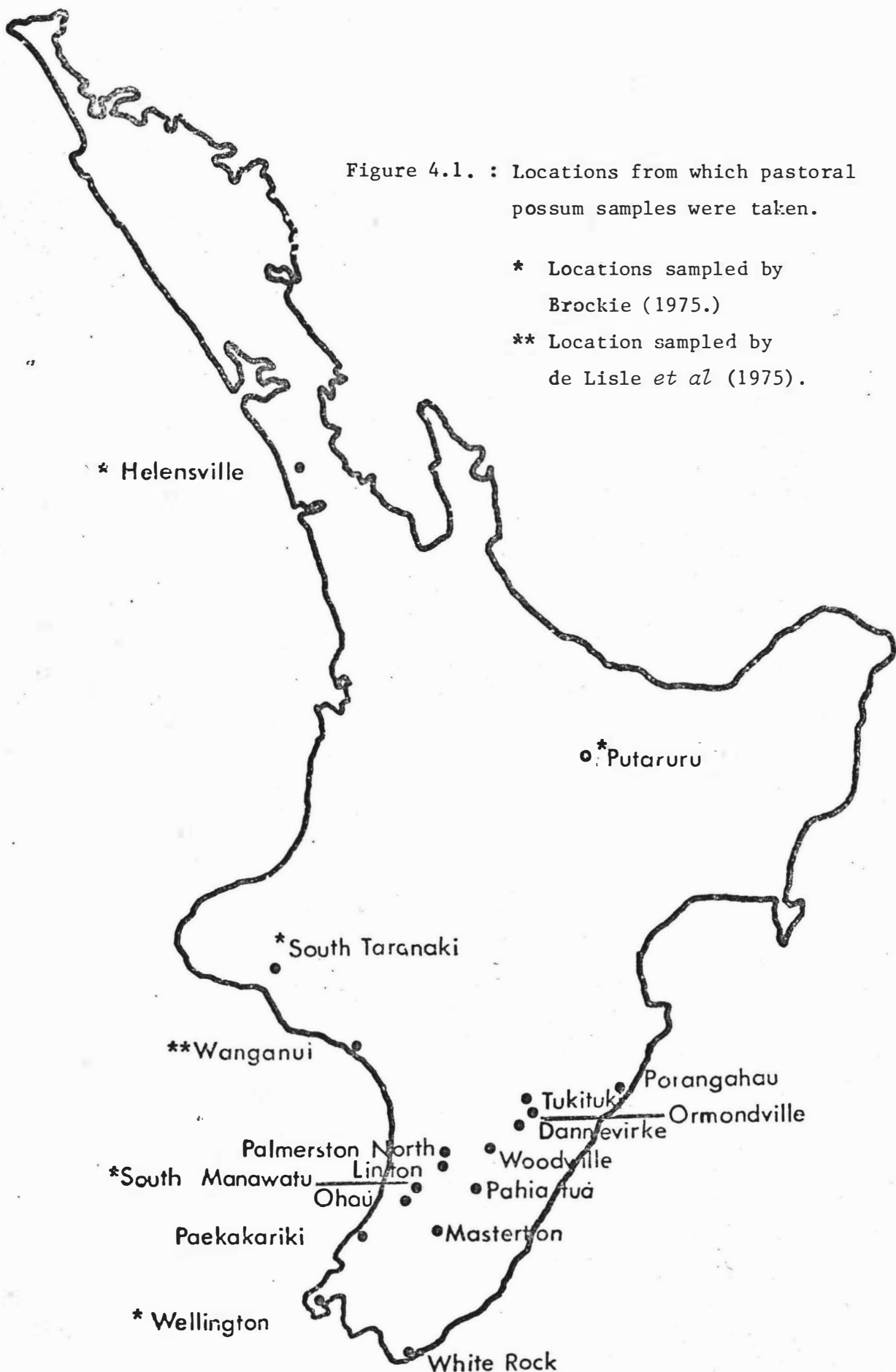
A comparison was made between sera from poisoned and shot possums by first heart-bleeding captive possums and then administering cyanide poison and recovering a blood sample from the carcass 12 hours later. In this way, paired blood samples from 12 adult possums were obtained and tested simultaneously in the MAT.

Sex ratios were calculated for possums collected by the three different sampling methods. Sex ratios have been shown to differ little from parity (Caughley, 1973; Jolly, 1973) and therefore any significant deviation from parity in the calculated ratios would indicate a bias in different sampling methods.

RESULTS

Sera selected at random for an estimate of the precision of the MAT had titres of from 0 to 1:3072. Results of the test for precision are given in Table 4.1. The difference in means of the two tests was non-significant ($t = 1.58$, $P > 0.1$).

A comparison of sera collected pre-mortem and then 12 hours post-mortem following cyanide poisoning showed that some differences occurred in titres of paired sera from the same animal (Table 4.3.). The difference in means of the sera sampled by the two methods was non-significant however ($t = 1.17$, $P > 0.05$).

As three different methods were employed to sample possum populations, the sex ratio was calculated for all possums collected by each method to detect any bias in sampling (Table 4.2.). No significant difference was found between the three methods ($Xc^2 = 2.14$, $P > 0.05$). The mean sex ratio for the three methods was not significantly different from parity ($Xc^2 = 1.52$, $P > 0.1$).

Table 4.1 : Precision estimate of titres of 100 sera tested
against *hardjo* antigen.

| Difference, in coded titre units * | -3 | -2 | -1 | 0 | +1 | +2 | +3 |
|---|---|---|---|---|---|---|---|
| Frequency | 1 | 4 | 17 | 45 | 24 | 6 | 3 |

* coded titre = $\log_2 \frac{X}{12}$

Table 4.2. : Sex ratios of possums collected by different sampling
methods.

| Method | Male:Female | Ratio |
|---|---|---|
| Night shooting | 220 : 188 | 1.17:1 |
| Trapping | 57 : 50 | 1.14:1 |
| Poisoning | 31 : 26 | 1.19:1 |

Table 4.3 : Comparison of titres in adult possums bled by cardiac
puncture pre-mortem and by jugular vein post-mortem.

| Possum No. | Reciprocal of titre to *hardjo* antigen | |
|:---:|:---:|:---:|
| | Cardiac puncture | Jugular vein  * |
| 1 | 0 | 0 |
| 2 | 96 | 48 |
| 3 | 1536 | 384 |
| 4 | 24 | 24 |
| 5 | 48 | 24 |
| 6 | 0 | 0 |
| 7 | 96 | 24 |
| 8 | 384 | 768 |
| 9 | 192 | 192 |
| 10 | 24 | 48 |
| 11 | 0 | 0 |
| 12 | 96 | 96 |

* sample collected following cyanide poisoning.

Serological results revealed a high prevalence of titres against *hardjo* antigen, therefore indicating a high prevalence of infection with one or more leptospires of the Hebdomadis serogroup (Table 4.4). Titres were present in samples from all pastoral localities, with a range in prevalence of from 41% to 65%. The mean prevalence of Hebdomadis serogroup titres over the 12 samples was 52%.

Very few titres against antigens other than *hardjo* were recorded (Table 4.5). Nine sera revealed titres against *ballum*, ranging from 1:24 to 1:384. These represented an overall prevalence of *ballum* titres of 1.5%. The geometric mean titre (G.M.T.) for positive *ballum* sera was 1:81. *Ballum* titres occurred in five sera which were negative to other antigens, and in the other four sera the titres to other antigens were lower than the titres to *ballum*. It is therefore considered that the *ballum* titres represented infection with a leptospire of the Ballum serogroup, rather than cross-reactions.

Two sera exhibited titres to *tarassovi* antigen at serum dilutions of 1:96 and 1:192. Two low titres to *copenhageni* (1:24 and 1:48) and three low titres to *pomona* (1:12, 1:24 and 1:96) were also recorded.

One titre of 1:192 against *biflexa* was recorded in an adult female and a titre of 1:24 to *pyrogenes* was recorded in an adult female with a *ballum* titre of 1:384. It was not known if these titres represented infection with these serovars or were cross-reactions.

Evidence of dual infection with leptospires from different serogroups, either concurrent or successive, was provided by the simultaneous occurrence of titres to *hardjo* and *ballum* in sera from four possums.

The prevalence of titres to *hardjo* antigen by sex, age-group and location is shown in Table 4.6. Adult males and females had a prevalence of 77% and 74% respectively and both transitional-stage males and females had a prevalence of 38%. Only 7% of juvenile males had positive *hardjo* titres. All the juvenile females were serologically negative.

The differences in prevalence between sexes within the mature adult, transitional-stage adult and juvenile age-groups were non-significant ($Xc^2 = 0.33$, P >0.75; $Xc^2 = 0.002$, P >0.9; $Xc^2 = 2.34$, P >0.7 respectively).

Table 4.4 : Prevalence of leptospiral titres to *hardjo* antigen in possums
from the southern half of the North Island of New Zealand.

| Location | No.possums examined | No.serologically positive | % serologically positive | S.E. of prevalence |
|---|---|---|---|---|
| Linton | 14 | 8 | 57 | 13 |
| Ohau | 12 | 5 | 42 | 14 |
| White Rock | 110 | 71 | 65 | 4.5 |
| Masterton | 40 | 25 | 63 | 7.5 |
| Woodville | 95 | 34 | 36 | 5 |
| Dannevirke | 48 | 20 | 42 | 7 |
| Ormondville 1 | 91 | 59 | 65 | 5 |
| Ormondville 2 | 46 | 22 | 48 | 6.5 |
| Tukituki | 7 | 3 | 43 | 19 |
| Porangahau | 54 | 34 | 63 | 6.5 |
| Pahiatua | 66 | 42 | 64 | 6 |
| Paekakariki | 17 | 7 | 41 | 12 |
| Total | 600 | 330 | 55 | 2 |

Table 4.5 : Titres to antigens other than *hardjo* in sera from pastoral
possum populations.

| Location | Class-ification | reciprocal of titre | | | | |
|---|---|---|---|---|---|---|
| | | *hardjo* | *ballum* | *copenhageni* | *pomona* | *tarassovi* |
| White Rock | JF | 0 | 0 | 0 | 0 | 96 |
| White Rock | LF | 0 | 0 | 24 | 0 | 0 |
| White Rock | LF* | 384 | 384 | 0 | 12 | 0 |
| White Rock | JF** | 96 | 24 | 0 | 0 | 0 |
| Masterton | F | 0 | 384 | 48 | 0 | 0 |
| Woodville | LM | 0 | 96 | 0 | 0 | 0 |
| Woodville | LM | 768 | 0 | 0 | 96 | 0 |
| Dannevirke | LM | 192 | 0 | 0 | 0 | 192 |
| Dannevirke | F | 0 | 24 | 0 | 0 | 0 |
| Dannevirke | LM | 0 | 0 | 0 | 24 | 0 |
| Ormondville 1 | LM | 96 | 192 | 0 | 0 | 0 |
| Tuki Tuki | LF | 0 | 24 | 0 | 0 | 0 |
| Porangahau | LF | 96 | 192 | 0 | 0 | 0 |
| Porangahau | LM | 0 | 24 | 0 | 0 | 0 |

** pouched joey of mature female*

Table 4.6.: Prevalence of titres to *hardjo* antigen,by sex, age-group and location,in pastoral possum populations

| Location | Males | | | Females | | | Trans.males | | | Trans.females | | | Juv.males | | | Juv.females | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No. | sero.+ve | % | No. | sero.+ve | % | No. | sero.+ve | % | No. | sero.+ve | % | No. | sero.+ve | % | No. | sero.+ve | % |
| Linton | 6 | 5 | 83 | 4 | 3 | 75 | 0 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 |
| Ohau | 4 | 2 | 50 | 3 | 3 | 100 | 1 | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 | 1 | 0 | 0 |
| White Rock | 35 | 32 | 91 | 23 | 21 | 90 | 13 | 10 | 76 | 10 | 7 | 70 | 14 | 1 | 7 | 15 | 0 | 0 |
| Masterton | 8 | 7 | 88 | 18 | 17 | 94 | 0 | 0 | 0 | 5 | 1 | 20 | 3 | 0 | 0 | 6 | 0 | 0 |
| Woodville | 33 | 17 | 51 | 30 | 14 | 47 | 7 | 1 | 14 | 3 | 1 | 33 | 9 | 1 | 11 | 13 | 0 | 0 |
| Dannevirke | 10 | 8 | 80 | 15 | 10 | 66 | 2 | 0 | 0 | 5 | 1 | 20 | 9 | 1 | 11 | 7 | 0 | 0 |
| Ormondville 1 | 29 | 23 | 78 | 39 | 29 | 74 | 12 | 4 | 33 | 6 | 3 | 50 | 4 | 0 | 0 | 1 | 0 | 0 |
| Ormondville 2 | 14 | 7 | 50 | 20 | 14 | 70 | 4 | 0 | 0 | 2 | 1 | 50 | 2 | 0 | 0 | 4 | 0 | 0 |
| Tuki Tuki | 2 | 2 | 100 | 1 | 2 | 50 | 2 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 |
| Porangahau | 27 | 23 | 85 | 10 | 8 | 80 | 7 | 3 | 43 | 3 | 0 | 0 | 2 | 0 | 0 | 5 | 0 | 0 |
| Pahiatua | 32 | 28 | 88 | 15 | 10 | 66 | 4 | 2 | 50 | 3 | 1 | 33 | 7 | 1 | 14 | 5 | 0 | 0 |
| Paekakariki | 4 | 3 | 75 | 5 | 4 | 80 | 3 | 1 | 33 | 1 | 0 | 0 | 2 | 0 | 0 | 2 | 0 | 0 |
| Total | 204 | 157 | 77 | 183 | 135 | 74 | 55 | 21 | 38 | 40 | 15 | 38 | 57 | 4 | 7 | 61 | 0 | 0 |

There was a marked trend of increasing prevalence of titres with age, with highly significant differences being found between juveniles and transitional-stage adults ($Xc^2$ = 50.00, P<0.005) and transitional-stage adults and mature adults ($Xc^2$ = 39.81, P<0.005). G.M.T's were calculated for each sex and age group and the results are presented in Table 4.7. Combined sexes in the transitional-stage adult age-group had a G.M.T. for positive sera of 1:467, the highest of the three age groups. Combined adult males and females had a G.M.T. for positive sera of 1:289 while that of combined juvenile males and females was much lower at 1:63.

A graphical representation of the relationship between the prevalence of *hardjo* titres and G.M.T's of positive sera and all sera within each sex and age-group is shown in Figure 4.2. Transitional-stage adults, with a prevalence of 38%, had a GMT for positive sera of 1:487 whereas mature adults, with a prevalence of 75%, had a much lower GMT for positive sera (1:289). The difference in GMT's for adult age-groups can be explained by reference to Figure 4.3. Transitional-stage adults had a skewed frequency distribution of *hardjo* titres towards the high titre range while the frequency distribution of titres for mature adults was approximately normal. Titres of juvenile animals occurred mainly in the low titre range.

When GMT's for all sera within each age-group are compared, it can be seen that the large difference in prevalence of titres between adult age-groups masks the effect of differences in frequency distribution of titres between age groups (Table 4.7). Thus, the GMT for all sera in the mature adult age-group is greater than the GMT for all sera in the transitional-stage adult age group.

The GMT's for positive sera from adult possums from different localities showed considerable variation. The 95% confidence limits of the GMT for each sample are shown in Table 4.8 (confidence limits for each GMT are assymetrical due to the logarithmic nature of a geometric mean transformation). Due to insufficient numbers, GMT's were not calculated for samples from Ohau, Linton, Tukituki and Paekakariki.

Table 4.7. : Prevalence and geometric means of *hardjo* titres, by age-group and sex, in sera from pastoral possums.

| Classification | Prevalence % | S.E.% | G.M.T. | |
|---|---|---|---|---|
| | | | Positive sera | All sera |
| JM | 7 | 3.5 | 63* | 14 |
| JF | 0 | 0 | 0 | 0 |
| JM & JF | 3 | 1.5 | 63 | 13 |
| TM | 38 | 6.5 | 489 | 47 |
| TF | 38 | 7.5 | 446 | 47 |
| TM & TF | 38 | 5 | 467 | 47 |
| LM | 77 | 3 | 269 | 132 |
| LF | 74 | 3 | 323 | 129 |
| LM & LF | 75 | 2 | 289 | 130 |

* reciprocal of titre

Table 4.8. : GMT's and 95% confidence limits for sera from adult possums with titres to *hardjo* antigen.

| Location | GMT and 95% confidence limits | | |
|---|---|---|---|
| White Rock | 545* | 740 | 1018 |
| Masterton | 125 | 229 | 427 |
| Woodville | 320 | 478 | 735 |
| Dannevirke | 141 | 269 | 523 |
| Ormondville 1 | 133 | 166 | 223 |
| Ormondville 2 | 151 | 182 | 218 |
| Porangahau | 266 | 388 | 615 |
| Pahiatua | 142 | 218 | 340 |

* reciprocal of GMT

Figure 4.2. : Prevalence of *hardjo* titres and geometric mean titres, by age-group and sex, in pastoral possums.*
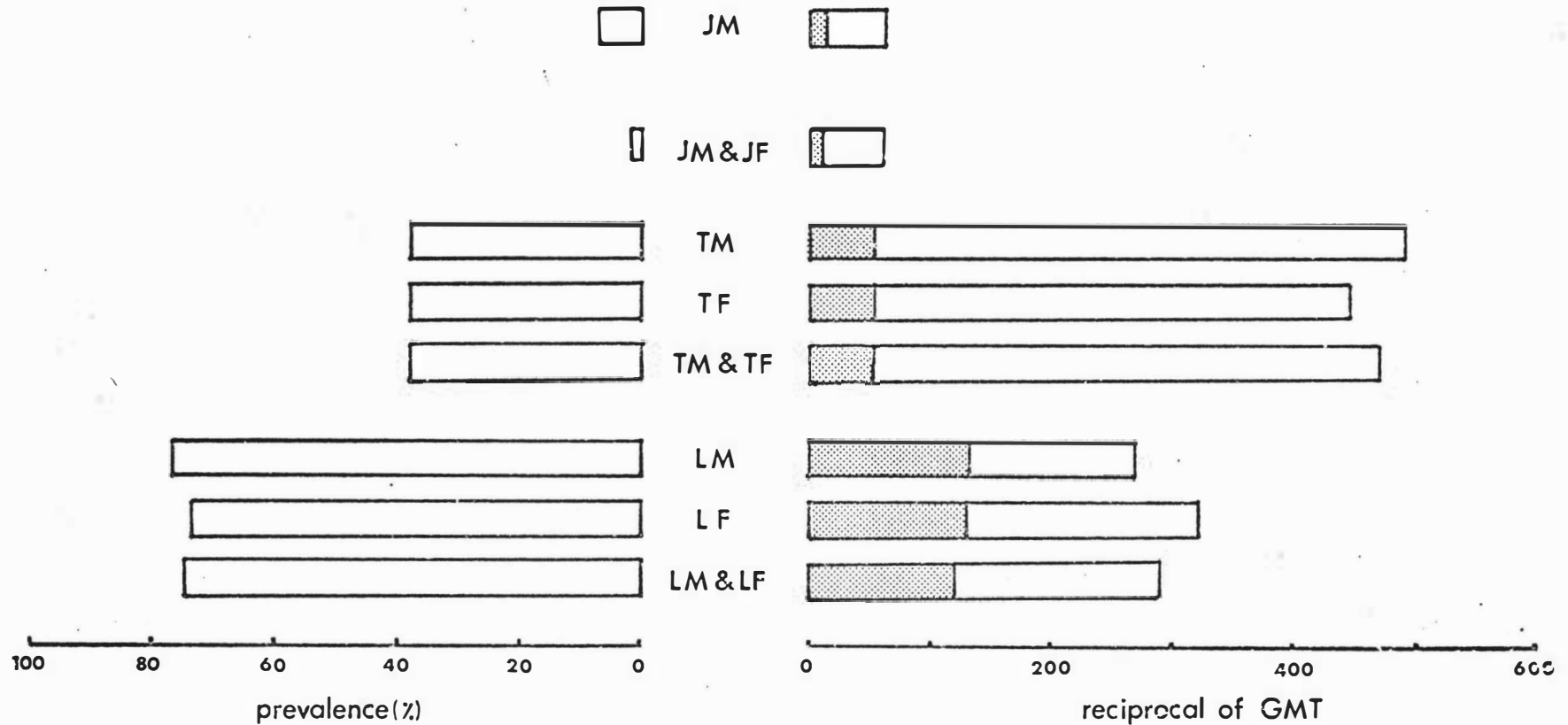
* excludes possums from Linton, Ohau, Tukituki and Paekakariki.

DISCUSSION

Infection of wildlife with leptospires of the Hebdomadis serogroup has been demonstrated in many countries (Parnas *et al*, 1961; Gordon-Smith *et al*, 1961; Emanuel *et al*, 1964; Clark *et al*, 1966; Torten *et al*, 1970; Mateev and Manev, 1974), and the worldwide distribution of organisms from this serogroup in leptospiral infection of domestic animals and man is well recognised.

The results of the present serological survey extend the findings of earlier New Zealand workers and show that Hebdomadis serogroup infection is widespread in pastoral possum populations in the southern half of the North Island. No predications as to the identity of the infecting serovar could be made from the titres against *hardjo* antigen as strong cross-reactions occur between members of the Hebdomadis serogroup (Alston and Broom, 1958; Manev and Yanakieva, 1973; Ellis and Michna, 1977).

The serological response to leptospiral infection has been well-defined by many authors, and is reviewed by Alston and Broom (1958) and Turner (1968). Peak titres are usually reached within three weeks of infection and then titres decline at a variable rate depending on species and infecting serovar. Titres have been reported to persist for from four to ten years in domestic cattle (Morse *et al*, 1955: Hanson, 1976) and may persist at low levels for up to 20 years in man (van der Hoeden, 1958).

The time for which titres persist following infection in wild animals is not well-defined. Several authors have stated that some species of wildlife, especially rodents, may remain infected for life (Babudieri, 1958; van der Hoeden, 1958; Roth, 1970; Tripathy and Hanson, 1976), however, there is little documented evidence of this lifetime carrier-state. Consequently, little is known about the long-term changes in titres following leptospiral infection in free-living animals. Short-term studies have shown that titres will persist for at least 197 days in striped skunks (Tabel and Karstad, 1967) and at least 20 months in chronically-infected gerbils (*Merinones unguiculatus*)
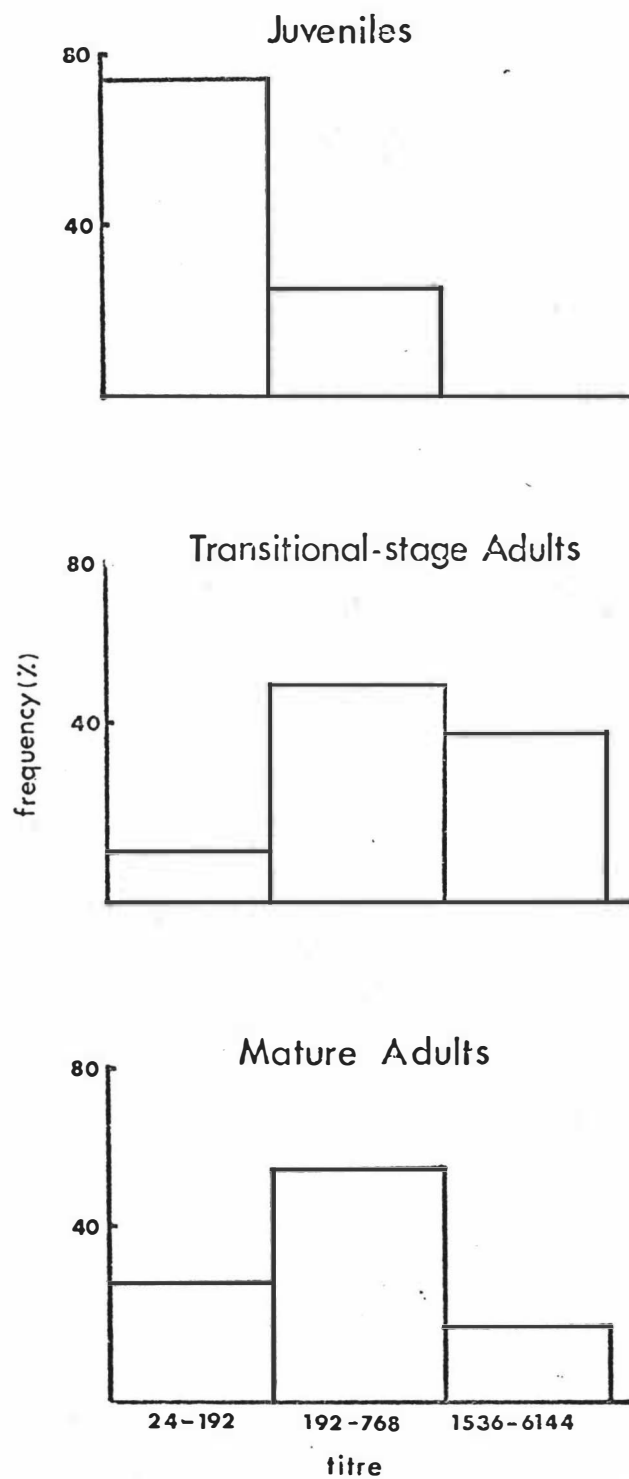
Figure 4.3. : Frequency distribution of *hardjo* titres by age-group in pastoral possums.

(Tripathy and Hanson, 1976). Other workers have reported that, in some species, a proportion of chronically-infected animals in the population have no demonstrable antibodies in their sera (Babudieri, 1958; Roth *et al*, 1963; Ferris and Andrews, 1967; Chernukcha *et al*, 1974). This situation is also recognised in domestic animals (Hirschberg and Vaughn, 1973; Ryan, 1978).

Titres recorded in the present survey had a range of from 1:24 to 1:6144 and the frequency distribution of titres in each sample was approximately normal. This distribution of titres, together with the high prevalence of titres found in all populations sampled, indicates that leptospiral infection is holoendemic in all pastoral possum populations in the southern half of the North Island. The relationship between serological and bacteriological prevalences is investigated in Chapter VI.

Leptospirosis in a free-living species is generally characterised by infection with more than one serovar (Parnas *et al*, 1961; Roth *et al*, 1963; Andrews *et al*, 1964; Torten *et al*, 1970; Fennestad and Borg-Petersen, 1972), although, in certain localities, some species (notably rodents) have been shown to be infected with one serovar only (Babudieri, 1958 ; Brown and Gorman, 1960; Chernukcha *et al*, 1975; Shotts *et al*, 1975). The almost exclusive restriction of titres to the Hebdomadis serogroup in possums in New Zealand is an important feature of leptospiral infection in this species. Titres to antigens from other serogroups were present in only 2% of sera tested and this very low prevalence is supported by the serological observations of previous workers (Salisbury, 1954; Brockie, 1975; de Lisle *et al*, 1975).

The very low prevalence of titres to antigens other than *hardjo* also demonstrates that cross-reactions are uncommon or do not occur in the sera from possums with Hebdomadis serogroup infections. Cross-reactions to a variety of antigens are common in sera from domestic animals with Hebdomadis serogroup infections (Murnane *et al*, 1963; Emanuel *et al*, 1964) but such cross-reactions appear to be uncommon in free-living species. Gordon-Smith *et al* (1961) found that sera with Hebdomadis titres from several rodent species in Malaya did not cross-react with other serogroup antigens. This situation was also noted in

Australian wildlife by Emanuel *et al* (1964). The lack of cross-
reactions in sera from possums with Hebdomadis serogroup infections
allows the presence of infection with leptospires from other sero-
groups to be more easily detected.

The finding of serogroup Ballum titres in the sera of nine
possums (1.5%) adds to the wide range of animal species that can be
infected with this serovar (see Chapter I). Organisms from the
Ballum serogroup have been isolated from hedgehogs, ship rats and
Norway rats in New Zealand and these free-living species may have
been the source of the sporadic *ballum* infections in possums.

The low GMT of 1:81 for sera with *ballum* titres is consistent
with serological observations in Ballum serogroup infections in other
free-living species. Several investigators have reported that titres
recorded in *ballum* infections are much lower than those recorded in
infections by leptospires from other serogroups (Alexander *et al*, 1963;
Roth *et al*, 1963; Shotts *et al*, 1975; Brockie, 1977). Therefore, these
low titres in possums may indicate present or recent infection at the
time of sampling.

Cross-reactions between Ballum and several other serogroups,
notably Icterohaemorrhagiae, Canicola and Pyrogenes, occur in a variety
of species (Alston and Broom, 1958; Ferris *et al*, 1961; Alexander *et al*,
1963). Although no consistent cross-reaction pattern with other sero-
group antigens occurred in possum sera with *ballum* titres, it is likely
that the titre of 1:48 recorded to *copenhageni* antigen was a cross-
reaction with the *ballum* titre for that serum of 1:384. Similarly, the
titre of 1:24 to *pyrogenes* recorded in a serum with a *ballum* titre of
1:384 was probably a cross-reaction.

The single titres to *tarassovi* and *biflexa* antigens and the three
low *pomona* titres are of unknown significance. These titres may have
represented infection with the homologous serogroup, as *tarassovi* and
*pomona* have been isolated from domestic animals in New Zealand (see
Chapter I) and possums sharing a pastoral environment may well contract
leptospirosis from this source. Serovar *andamana,* a member of the
saprophytic Biflexa complex, has recently been isolated from two Norway
rats in Israel (Shenberg *et al*, 1975) and therefore its classification

as a saprophyte is open to question. Such a serovar from the Biflexa
complex may have been responsible for the *biflexa* titre recorded in a
possum in the present survey.

Titres to both *ballum* and *hardjo* antigens were found in the sera
from three adult possums and were considered to be evidence of dual
infection by leptospires from different serogroups. These dual
infections may or may not have occurred concurrently. Further evidence
of dual infection was provided by the low *hardjo* and *ballum* titres found
in the serum of a pouched joey whose dam had titres of 1:384 to *hardjo*
and *ballum*. It is probably that passive transfer of immunoglobulins
from the dam resulted in the dual titres being recorded in the joey.
Dual leptospiral infection has been reported in several free-living
species (Gordon-Smith *et al*, 1961; Roth *et al*, 1963; Ferris and Andrews,
1964).

The prevalence of *hardjo* titres in males and females was very
similar. This situation has been found in most leptospiral investigations
although sex-specific prevalences have been shown to be significantly
different in studies of some free-living species (Blood *et al*, 1963;
Twigg *et al*, 1968; Fennestad and Borg-Petersen, 1972). These authors
were unable to explain these differences in prevalences between males
and females.

Several workers have found a marked difference in age-specific
prevalence of leptospiral infection in free-living populations. Ferris
*et al* (1961) recovered *ballum* only from adults from a mixed-age sample
of 87 house mice and Shotts *et al* (1975) recovered leptospires from
only adults in a mixed-species sample of 144 small wild mammals.
Similar results have been reported in hedgehogs (Wolff and Bohlander,
1965), Norway rats (Kallai *et al*, 1962) and Pampas cavies (*Cavia pamparum*)
(Blood *et al*, 1963). However, other investigators have reported little
difference in prevalence of infection between the juveniles and adults
of some free-living species (Ferris and Verts, 1964; Twigg *et al*, 1968).
As age-differentiation of animals is not carried out in many leptospiral
surveys of wildlife, information on age-specific prevalence of infection
is often not available.

In the present survey, the very low prevalence of titres in juvenile possums was a consistent observation in all populations sampled. The few titres recorded (4 out of 117 sera tested) were low and restricted to animals weighing less than 600 grams and it is probable that these were residual maternal antibody titres. The passive transfer of immunoglobulins from dam to off-spring is investigated in Chapter VIII.

Transitional-stage adults had a prevalence of *hardjo* titres of 38%, considerably lower than that of mature adults (75%). Thus, the highly significant difference in age-specific prevalences is an important feature of leptospiral infection in pastoral possums. Any comparison between prevalences of infection in populations from different localities therefore requires that the age-structure of the population be known. As mature adults constitute the majority of animals in a population and also experience the highest prevalence of infection, these animals provide the best groups for comparison of prevalences of infection. The samples collected by Brockie (1975) and de Lisle *et al* (1975) were not differentiated as to age and therefore the prevalence of infection recorded in these surveys cannot be compared with those recorded in the present study.

The transitional-stage adult group had a considerably higher GMT for positive sera than that of other age-groups and this was considered to be indicative of a more recent time of infection than that occurring in other age-groups. The frequency distribution of titres in this age-group, with most titres being in the mid and high range, illustrates this hypothesis. The mature adult age-group, with a higher prevalence of titres, had a frequency distribution of titres skewed towards the low-titre range, indicative of a greater percentage of animals with an older time of infection. The low GMT of titres recorded in the juvenile age-group was consistent with the probability that these titres were due to residual, maternally-derived antibody. The highly significant difference in age-specific prevalence of leptospiral titres in possums is discussed relative to bacteriological prevalence in Chapter VI.

GMT's for all sera in each group were calculated as well as GMT's for positive sera. As already discussed, differences in prevalence of titres between groups markedly influenced the GMT for all sera compared

with the GMT's for positive sera. This parameter was therefore of little use when comparing different age-groups and consequently, throughout the rest of the study, only GMT's for positive sera are calculated.

Although variation in prevalence of titres and GMT's for positive sera occurred between mature adult groups from different pastoral localities, this variation could not be correlated with location or type of farming. Therefore it appeared likely that population factors were the more important determinants of the disease status of each possum population sampled.

The eight pastoral localities for which the prevalence of titres and GMT's of positive sera in mature adults were compared all supported moderate or high population densities, as assessed by spotlight counts. The two locations where densities were high (White Rock and Woodville) had the highest GMT's for positive sera, however, the prevalence of titres in mature adults from Woodville was much lower than that from White Rock. Variation also occurred in the prevalence of titres and GMT's of positive sera in populations from areas of similar population density. Therefore the variation in prevalence of titres in pastoral possum populations did not appear to be closely related to population density. This observation could not be fully evaluated from this initial survey for several reasons. Spotlight counts are subjective and are often inaccurate and therefore only gross differences in population density can be detected. As a consequence, estimates of population density can only be placed into broad categories. In addition, prevalences for mature adults were all higher than 47% and usually higher. When taking into account the problem of sampling error, a correlation between a subjective estimate of population density and the prevalence of titres would be hard to establish, because of the lack of contrast in the differences between populations sampled in this survey.

It is also likely that the age structure of a population is a factor which affects the prevalence of titres and the GMT of positive sera from mature adults. Populations that are subjected to commercial exploitation have a higher proportion of younger animals in the mature adult group than unexploited populations. If the majority of possums

become infected by the end of their third year, higher GMT's would be expected in exploited populations with a young age structure. An indication of the age structure of a population can be gained from adult:juvenile ratios. Although in the present study the regression of log GMT on the adult:juvenile ratio of different populations was non-significant (y = 2.67-0.04x, t = 2.00, P>0.05), the population with the highest GMT (White Rock) had a low adult:juvenile ratio (2.8).

The comparison between blood-sampling techniques used in the initial survey showed that although there was some individual variation in paired titres, the difference between the means of the samples collected by the two techniques was not significant. They were therefore proved to be satisfactory techniques for comparative analysis and were used throughout the remainder of the study.

The absence of a sex bias between the three sampling methods used demonstrated that representative samples were being obtained from populations, regardless of the method used. The bias towards an excess of females over males that was observed by Tynedale-Biscoe (1955), when night-shooting was compared with trapping, was not evident in this survey.

There was no significant difference in coded titre means between the two tests on sera used for an estimate of the precision of the MAT, and 86% of titres recorded in the second test were with ± one serum dilution of the titres recorded in the first test. This level of precision is slightly lower than the 90% of titres within ± one dilution reported in a precision estimate carried out by Hellstrom (1978). A slightly skewed distribution of titres recorded at the second test relative to the first may have been due to the use of an antigen of lesser density, however, Graves and Faine (1970) reported that up to a 40% variation in antigen did not significantly alter the precision of the MAT in their laboratory.

## SUMMARY AND CONCLUSIONS

1. The prevalence of Hebdomadis serogroup titres is very high in pastoral possum populations in the southern half of the North Island of New Zealand.

2. Titres were almost exclusively restricted to the Hebdomadis serogroup, indicating that infections with leptospires from other serogroups were rare.

3. Evidence of infection with leptospires of the Ballum serogroup was present in 1.5% of sera tested.

4. There was a highly significant difference in age-specific prevalence of titres in all populations sampled.

5. The highest GMT was found in the transitional-stage adult age-group, indicating that this group had experienced infection more recently than the mature adults.

6. No significant difference was shown between the means of titres recorded in sera collected *pre-* or *post-mortem*.

7. An estimate of the precision of the MAT showed that 86% of titres were within ± one serum dilution of the initial test.

## CHAPTER V

## DEVELOPMENT OF CULTURAL TECHNIQUES

### INTRODUCTION

The use of serology for the diagnosis of leptospiral infection in animals has serious limitations (van der Hoeden,1958; Roth,1970; Turner,1970), and therefore an efficient cultural method is an important prerequisite of any epidemiological study. The isolation of leptospires from host tissue and fluids is affected by many factors and a wide variety of methods have been used to isolate or demonstrate infecting organisms (see Chapter III). The relative efficiency of the methods used in this study, as applied in the initial cultural survey of 141 animals and in specific laboratory investigations, is described in this chapter. As a consequence of these findings, some changes were made in the cultural techniques using during the remainder of the study.

### MATERIALS AND METHODS

The materials and general methods used in this chapter are described in Chapter III.

A total of 141 kidneys were cultured in semisolid EMJH and 103 of these kidneys were also cultured in semisolid EMJH containing 200 μg 5FU/ml. Comparative isolation and contamination rates were calculated for each dilution of kidney homogenate.

The effect of time of culture after death on the recovery rates of leptospires was investigated by the comparative culture of each kidney from each of 34 possums. One kidney from each animal was cultured within one to three hours of death the the other was left in the carcass and cultured within 18 - 24 hours of death. The number of isolations made from each kidney and the number of contaminated cultures were then compared.

Silver-impregnated kidney sections for histological examination were prepared from 33 adult possums. Two sections from each kidney

were examined.

An attempt was made to recover leptospires from the urine
of 28 possums by direct inoculation of media in the field followed
by serial subculturing in the laboratory 24 hours later to remove
contaminants.  Urine samples were also filtered in an attempt to
prevent contamination of cultures.  Urine samples were cultured in
media without 5FU and media with 200 µg 5FU/ml.

Darkfield examinations were carried out on urine samples from
16 possums collected close to Massey University.  Cultures were
made from the cerebral cortex of these animals also.

An experiment was carried out to determine the relative
efficiency of media, with and without a selective inhibitor, for
the isolation of leptospires from different serial dilutions of
voided urine, urine aspirated from the bladder and kidney homogenate.
The transitional-stage adult possum used for this experiment was
experimentally infected with serovar *balcanica* and, at the time
of culture, was excreting $2.25 \times 10^6$ organisms/ml of voided urine.
The number of leptospires was estimated by calculating the mean
of three counts of urine in a Petroff-Hauser counting chamber.  An
initial kidney homogenate was prepared in Stuarts base medium (SBM)
(1:5 w/v) and six serial ten-fold dilutions of this homogenate were
made in SBM.  Five tubes of semisolid EMJH were inoculated with 0.25ml
of each dilution of homogenate and this was repeated with a series
of semisolid media containing 200 µg 5FU/ml and a series containing
400 µg 5FU/ml.  The same dilutions and inoculations were made for
voided urine and urine aspirated in a sterile manner from the
bladder.  In addition, two weanling hamsters were inoculated with
each dilution of voided urine.

As weanling hamsters were used extensively throughout the
study for the isolation of leptospires, an experiment was carried
out to determine the minimum infective dose (MID), minimum lethal
dose (MLD) and most efficient route of infection for two serovars
from different serogroups.  Recently isolated strains of *balcanica*
and *pomona* were diluted in SBM to produce serial ten-fold dilutions

of leptospires of from $5 \times 10^6$ to $5 \times 10^1$ organisms/ml. Six groups of eight hamsters were each inoculated intraperitoneally with 0.1 ml of each dilution of culture. This procedure was repeated using the percutaneous route. Inoculations by this route were made by anaesthetising the hamsters with ether, and abraiding the ventral abdomen with a scalpel blade until hyperaemic. A small adhesive template was applied to the skin and 0.05mls of an appropriate culture dilution placed in the centre. Anaesthesia was maintained until the drop had dried. The template was then removed and the animal allowed to regain consciousness.

The kidneys of hamsters that died following inoculation were cultured by standard techniques. The experiment was terminated at 21 days and kidneys of survivors at this time were also cultured.

All isolates obtained from field cultures were grown in liquid medium until of sufficient density for typing. A small proportion of isolates however were difficult to grow when subcultured from primary semisolid medium (0.15% agar) into liquid medium. In an attempt to facilitate this passage, an intermediate subculture was carried out in semisolid medium containing 0.05% agar.

RESULTS

The effect of dilution of kidney homogenate on isolation rates is shown in Table 5.1. Fifty-two isolations were made from the $10^{-1}$ dilution (37%), and slightly fewer were made from the $10^{-2}$ and $10^{-3}$ dilutions. This trend was non-significant ($X^2 = 0.62$, P >0.5). Three isolations were made from the $10^{-2}$ dilution that were not made from the $10^{-1}$ dilution and no isolations were made from the $10^{-3}$ dilution that were not made from either the $10^{-1}$ or $10^{-2}$ dilutions. The combined isolation results from the $10^{-1}$ and $10^{-2}$ dilutions of homogenate gave a total of 55 isolations from 141 kidneys cultured (39%).

The trend of decreasing number of isolations as homogenate dilution increased that was observed in media without 5FU was also apparent in media containing 200 µg 5FU/ml. This was also non-

significant ( $X^2 = 1.81$, P $>$ 0.25 ).  A total of 38 isolations were made from 103 kidneys cultured in this medium (37%).  One isolate recovered from the $10^{-2}$ dilution of kidney homogenate was not recovered from the $10^{-1}$ dilution.  As with media not containing 5FU, no isolations were made from the $10^{-3}$ dilution of a particular homogenate that were not made from either the $10^{-1}$ or $10^{-2}$ dilution.

Chi-squared analysis of the number of isolations obtained from the two types of media showed that the slightly higher percentage from media without 5FU was not significantly different from the percentage from media containing 200 µg 5FU/ml ( $X^2 = 0.04$, P $>$ 0.75).

Of the kidney homogenates that were cultured in both types of medium (73%),in four cases isolations were made in media without 5FU that were not made in media containing 5FU.  In contrast, two isolations were made in media containing 200µg 5FU/ml that were not made in media without this selective inhibitor.

The overall contamination rate of kidney cultures was very low, with only 23 of 732 tubes inoculated being contaminated (3.1%)(Table 5.2).  The contamination rate in media containing 200 µg 5FU/ml (1.5%) was significantly lower than that in media without 5FU (4.5%)( $X^2 = 4.87$, P<0.05).

Contamination occurred sporadically in cultures of all kidney homogenate dilutions.  In only three cases were all cultures in one type of media from one kidney contaminated.  These occurred in media without 5FU.

The investigation into the effect that time of culture after death had on the number of isolations made showed that more isolations were obtained when kidneys were cultured within three hours of death compared with kidneys which were cultured 18 - 24 hours after death (Table 5.3).  Contamination occurred in the culture series from four kidneys cultured at the later time compared with no contamination of the culture series of kidneys cultured within three hours of death.  An interesting observation in this investigation was the recovery of two isolates from kidneys cultured at 18 to 24 hours that were not made from kidneys from the

Table 5.1 : Effect of dilution on recovery rates of leptospires, using media with and without 5-fluorouracil

| Media | No. kidneys cultured | No. Isolations (%) | No. isolations from each homogenate dilution | | |
|---|---|---|---|---|---|
| | | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
| EMJH | 141 | 55 (39) | 52 | 47 | 45 |
| EMJH + 200μg 5FU/ml | 103 | 38 (37) | 37 | 33 | 28 |

Table 5.2 : Contamination rates of media with and without 5-fluorouracil

| Media | No. tubes inoculated | No. tubes contaminated (%) | No. tubes contaminated from each homogenate dilution | | |
|---|---|---|---|---|---|
| | | | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ |
| EMJH | 423 | 19 (4.5) | 8 | 5 | 6 |
| EMJH + 200 μg 5FU/ml | 309 | 4 (1.3) | 2 | 1 | 1 |

Table 5.3 : Leptospiral isolations from comparative culture of
kidneys at two different time intervals post-mortem.

| Culture data | Time of culture (hrs). | |
|---|---|---|
| | 1 - 3 | 18 - 24 |
| No. kidneys cultured | 34 | 34 |
| No. isolations | 11 | 8 |
| No. isolations made at one time of culture only | 5 | 2 |
| No. contaminated culture series | 0 | 4 |

same animals cultured within three hours of death.

Silver-impregnation of kidney tissue proved to be a poor
method of demonstrating leptospiral infection in possums. Of
33 kidneys stained by a modified Warthin-Starry technique, lepto-
spires were seen in sections from only two animals (6%). Isolations
from kidney homogenates were made from 17 possums in this group
(51.5%).

Culture of urine was seriously hampered by heavy contamination
of a large proportion of cultures (Table 5.4). Contamination
occurred in 65 of 72 tubes containing media without 5FU (90%).
Dilution of urine had little effect on reducing contamination rates
and attempts to remove contamination by serial subculture resulted
in only 14% of contaminated cultures being cleared. Fewer contamin-
ated urine cultures occurred in media that contained 200 µg 5FU/ml
and there was a significant decrease in contamination of cultures
with increasing dilution of urine inocula ($X^2$ = 15.35, P< 0.005).
Due to high contamination rates however, both types of media were
unsatisfactory for the isolation of leptospires from urine.

Filtration was effective in removing contaminating bacteria
from urine (Table 5.4). Only two of 24 tubes inoculated with neat
filtered urine were contaminated (8%). No contamination occurred
in cultures that had been inoculated with diluted urine that had
been filtered. Despite the absence of contamination, no isolations
were made from filtered urine and this suggested that filtration had
also removed leptospires from the inocula.

Two isolations were made from the 24 possum urines cultured
(8%) and this low isolation rate was a reflection of the large num-
ber of contaminated cultures. In comparison, isolations were made
from kidney homogenates of 11 of the 24 animals from which urine
was cultured (46%).

Poor results were also obtained from dark-field examination of
urine samples. Leptospires were seen in the urine of only one of 17
mixed-age possums (6%) and no leptospires were seen in centrifuged
urine samples. Culture of kidneys from this group produced six

Table 5.4 : Contamination rates of urine cultures

| Media | No. urine samples | Number cultures contaminated | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | Urine dilution | | | Filtered urine dilution | | |
| | | Neat | 1:50 | 1:500 | Neat | 1:50 | 1:500 |
| EMJH | 24 | 24(100)[a] | 22(92) | 19(79) | 2(8) | 0 | 0 |
| EMJH + 200 µg 5FU/ml | 24 | 20( 83) | 16(67) | 7(29) | NT | NT | NT |

[a] percentage in brackets.

Table 5.5 : Isolations of leptospires from urine cultures.

| Media | No. urine samples | Number of isolations | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | Urine dilution | | | Filtered urine dilution | | |
| | | Neat | 1:50 | 1:500 | Neat | 1:50 | 1:500 |
| EMJH | 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| EMJH + 200 µg 5FU/ml | 24 | 1 | 2 | 1 | NT | NT | NT |

Table 5.6 : Diagnosis of leptospiral infection in possums by different test methods relative to kidney culture.

| Diagnostic Method | No. tested | No. positive | % positive | No. positive by kidney culture | % positive by kidney culture | Sensitivity of of test method (%) |
|---|---|---|---|---|---|---|
| Kidney sections | 33 | 2 | 6 | 17 | 52 | 12 |
| Urine culture | 24 | 2 | 8 | 11 | 46 | 18 |
| Dark-field urine examination | 17 | 1 | 6 | 6 | 35 | 17 |

isolates (35%). No isolations were made from cultures of brains from possums.

The results of the experiment to determine the relative efficiency of different types of media to isolate organisms from different dilutions of voided urine, urine aspirated from the bladder and kidney homogenate are given in Tables 5.7 and 5.8. The high contamination rate that occurred in cultures of urine in media without 5FU made in the field also occurred in this experiment. Serial ten-fold dilutions of urine decreased contamination rates, but, at the level of dilution needed to remove contaminants, very few leptospires remained in the inocula (Table 5.7).

The incorporation of 200 µg 5FU/ml in media resulted in a marked decrease in contamination of urine cultures and, as a consequence, isolations were made from inocula containing as few as 55 organisms. No contamination of cultures occurred in media containing 400 µg 5FU/ml. The number of isolations made in this medium was very similar to the number made in media containing 200 µg 5FU/ml. (Table 5.7).

The MID and MLD for hamsters inoculated with urine containing leptospires was 5500 organisms (Table 5.7). Hamsters surviving for 21 days following inoculation were cultured and one hamster that survived an inoculation of neat urine was found to be infected. The kidney cultures of all hamsters that received $\leqslant 550$ organisms were negative.

Contamination was only sporadic in cultures of urine aspirated from the bladder (Table 5.8). Isolations were obtained from all urine dilutions except $10^{-6}$. By assuming that the concentration of organisms in urine from the bladder was the same as that in voided urine, organisms were isolated from inocula theoretically containing only five organisms. This was achieved in media without 5FU and in media containing 200 µg 5FU/ml.

Fewer isolations were made from high dilutions of urine

Table 5.7 : Isolation of leptospires from serial dilutions of
voided urine, using media with and without a
selective inhibitor.

| Voided urine dilution | No. organisms inoculated | Media | | | Hamster inoc. |
|---|---|---|---|---|---|
| | | EMJH | EMJH+200 µg 5FU/ml | EMJH+400 µg5FU/ml | |
| Neat | $5.5 \times 10^5$ | $0^a$ (5C)$^b$ | 3(2C) | 5 | $1^c$ |
| $10^{-1}$ | $5.5 \times 10^4$ | 0 (5C) | 4(2C) | 5 | 2 |
| $10^{-2}$ | $5.5 \times 10^3$ | 0 (5C) | 5 | 4 | 2 |
| $10^{-3}$ | $5.5 \times 10^2$ | 1 (4C) | 5 | 4 | 0 |
| $10^{-4}$ | $5.5 \times 10^1$ | 0 (2C) | 3 | 2 | 0 |
| $10^{-5}$ | 5.5 | 0 (2C) | 0 | 0 | 0 |
| $10^{-6}$ | 0 | 0 | 0 | 0 | NT |

[a] Number of isolations per five tubes inoculated

[b] Number of cultures contaminated per five tubes inoculated

[c] Number of deaths per two hamsters inoculated.

($10^{-3}$, $10^{-4}$ and $10^{-5}$) cultured in media containing 400 µg 5FU/ml compared with the same dilutions cultured in media containing 200 µg 5FU/ml. This difference was not significant ($Xc^2 = 3.34$, $P > 0.05$).

Isolation rates were highest and contamination rates lowest for cultures made from kidney homogenate. The three media used had similar ability to isolate leptospires from all dilutions of homogenate except $10^{-6}$ (Table 5.8). Two isolations made from the $10^{-6}$ dilution in media without 5FU were not made in media containing 5FU. Isolations from this dilution imply that a minimum of $4 \times 10^{-6}$ organisms were present in each ml of undiluted homogenate. It is probable that there were at least $2 \times 10^{7}$ organisms/ml of undiluted homogenate if it is accepted that an inoculum of five organisms is necessary for the recovery of leptospires in culture media. Dark-field examination of the kidney homogenate failed to detect this high concentration of leptospires and leptospires were not seen in two silver-impregnated sections from this kidney.

A comparison of the number of isolations made from the high dilution of the three host materials cultured shows that the most suitable material for the isolation of leptospires was kidney homogenate. The most sensitive medium for isolation of leptospires from non-contaminated sources was that which did not contain 5FU. When the number of organisms in the inoculum was small, there was a slight decrease in the number of isolates obtained from media containing 400 µg 5FU/ml compared with media containing 200 µg 5FU/ml. This trend was non-significant ($Xc^2 = 2.21$, $P > 0.25$).

Hamsters infected with *balcanica* exhibited clinical signs of severe central nervous disturbance, with circling and hyperaesthesia being especially apparent. Terminally, the animals showed clonic muscle spasms, became moribund and died within 48 hours of first showing clinical signs of infection. Only one hamster recovered after showing clinical signs of disease. This animal, which received 5,000 leptospires IP, was culture-positive at 21 days.

The MID and MLD for hamsters infected by different routes are shown in Table 5.9. The occurrence of asymptomatic infection with *balcanica* was demonstrated by the isolation of the organism from two of eight hamsters that survived an infective dose of 500 leptospires by the IP route. The MID for *balcanica* by the IP route was 50 organisms. Serovar *pomona* was more virulent for hamsters than *balcanica*. All

Table 5.8 : Isolation of leptospires from serial dilutions of
urine aspirated from the bladder and kidney homogenate,
using media with and without a selective inhibitor.

| Culture fluid | Dilution | EMJH | EMJH +200µg 5FU/ml | EMJH +400µg 5FU/ml |
|---|---|---|---|---|
| | Neat | $4^a$ $(1C)^b$ | 4 (1C) | 5 |
| | $10^{-1}$ | 5 | 5 | 5 |
| Bladder urine | $10^{-2}$ | 5 | 5 | 5 |
| | $10^{-3}$ | 4 (1C) | 5 | 3 |
| | $10^{-4}$ | 1 (2C) | 5 | 2 |
| | $10^{-5}$ | 2 (1C) | 1 | 0 |
| | $10^{-6}$ | 0 | 0 | 0 |
| | Neat | 5 | 5 | 5 |
| | $10^{-1}$ | 5 | 5 | 5 |
| Kidney homogenate | $10^{-2}$ | 5 | 5 | 4 |
| | $10^{-3}$ | 5 (1C) | 5 | 5 |
| | $10^{-4}$ | 4 | 5 | 5 |
| | $10^{-5}$ | 5 | 4 | 4 |
| | $10^{-6}$ | 2 | 0 | 0 |

[a] Number of isolations per five tubes inoculated

[b] Number of cultures contaminated per five tubes inoculated.

*pomona* deaths occurred within seven days of inoculation compared with *balcanica* where some deaths were not recorded until 18 days post inoculation. Only one hamster survived an infecting dose of *pomona* of ≥ 50 leptospires by the IP route. This animal was culture positive at 21 days and therefore non-lethal infection occurred in hamsters infected with *pomona* as well as in hamsters infected with *balcanica*.

The MID and MLD for both serovars were higher by the PC route than the IP route and therefore this route was not as efficient at establishing leptospiral infection as the IP route.

The growth of isolates that were difficult to establish in liquid media appeared to be improved by an intermediate passage in 0.05% semisolid agar between primary semisolid culture (0.15% agar) and liquid culture.

## DISCUSSION

The use of a simple whole-kidney homogenation technique was shown to be an efficient and sensitive method for the isolation of leptospires. Isolations were made from 55 of 141 kidneys cultured (39%). This was a much higher rate of recovery than that recorded in an earlier field report by Brockie (1975). In this study, a kidney plug technique was used and isolates were obtained from only two of 146 possums (1.4%).

With the use of sterile disposable plastic bags in the Coleworth Stomacher, whole kidneys were homogenised rapidly, efficiently and with a minimum of contamination. The apparatus was especially suited to the processing of fresh samples in the field, as it could be very easily operated in situations where there was a high risk of environmental contamination. Although the Stomacher was designed for the bacteriological examination of meat samples, the present results illustrate another important use of this apparatus.

Contamination occurred sporadically in cultures from all dilutions of kidney homogenate and was attributed to airborne contamination during the dilution and inoculation process, rather

Table 5.9 : Comparative data on experimental infection of
hamsters with serovars *balcanica* and *pomona*.

| Serovar | Route of Infection | MID | MLD | Mean death time (days) | Symptoms |
|---|---|---|---|---|---|
| *balcanica* | IP | 50 | 500 | 13.2 | + |
| *balcanica* | PC | 500 | 5000 | 14.6 | + |
| *pomona* | IP | 50 | 50 | 5.0 | − |
| *pomona* | PC | 500 | 500 | 5.6 | − |

than contamination of the primary kidney homogenate. The very low level and sporadic nature of contamination indicated that contamination did not adversely affect the isolation rate of leptospires. This is of major importance in the culture of leptospires from field samples.

Concentrated kidney homogenates were not found to be inhibitory to the growth of leptospires, as has been suggested by some workers (Ryu , 1965; Shotts,1976). In this study the number of isolations decreased as the dilution of homogenate increased and this probably reflected the smaller number of organisms present in the inocula from the $10^{-2}$ and $10^{-3}$ dilutions. As no isolations were made from the $10^{-3}$ dilution that were not made from either the $10^{-1}$ or $10^{-2}$ dilution, the culture of this dilution was discontinued during the remainder of the study.

The finding that a proportion of leptospires will remain viable in kidneys for at least 24 hours has also been demonstrated in other species (Michna, 1970; Ryan, 1978). The decrease in number of isolations with increasing time of culture after death may have contributed in part to the low number of leptospires isolated from possums by Brockie (1975). This investigator cultured kidneys the day following night-shooting operations.

Poor results were achieved from histological examination of kidneys. Similar results using this technique have been reported in other surveys (Bloom, 1961; Gordon-Smith *et al*,1961; Turner, 1970) and may reflect the stage of infection that exists in the majority of animals in a population with endemic leptospirosis. Bloom (1961) and Taylor *et al* (1970) reported that leptospires were much more difficult to find in kidney sections from dogs with chronic infection compared with dogs in the acute stage of infection. The failure to detect leptospires in the kidney of a possum excreting $2.25 \times 10^6$ organisms per ml of urine also illustrates the lack of sensitivity of this technique.

Brain cultures were unsuccessful. It has been suggested that leptospiral infection can persist in the brain of carrier animals (Langham *et al*, 1958; Sulzer and Jones,1974), however

this did not appear to be the case in the possums examined by this method.

The culture of leptospires from urine is affected by several factors. Leptospiruria may be constant in some species (McGowan and Karstad,1965; Hodges,1977) but is more often reported as being intermittent (Ferris and Andrews, 1967; Minette and Shaffer,1968; Sullivan, 1970; Ellis and Michna,1977). The duration of leptospiruria is also very variable, both within species (Webster,1955; Morse *et al*,1958) and between species (McGowan and Karstad,1965; Roth, 1970; Chernukcha *et al*,1974; Hanson, 1976). It has also been shown that leptospires may be isolated from kidneys long after detectable urinary shedding has ceased (Morse *et al*, 1958; Tabel and Karstad,1967; Chernukcha *et al*, 1974). Because of the above-mentioned factors, it can be expected that,in a cultural survey, fewer isolations will be made from urine samples than from kidneys. This was the case in the present survey, where urine isolates were obtained from only 18% of animals from which isolates were obtained from kidneys.

In addition to factors affecting leptospiruria,there are several factors that affect the viability of leptospires present in urine. These include pH (Noguchi,1918; Okazaki and Ringen,1957), time of culture after urine sampling (Kirschner and McGuire,1957; Nervig and Ellinghausen,1978), presence of leptospiral agglutinins (Morse *et al*,1958; Killinger *et al*,1976; Hanson, 1977), and contamination with other micro-organisms (Johnson and Rogers,1964; Hussaini and Ruby,1976). The relative importance of each of these factors on the viability of leptospires is contentious, however all workers agree that contamination of cultures severely limits the recovery of leptospires from urine.

The present study showed that media without 5FU and media containing 200 μg/ml 5FU were unsuitable for culture of possum urine, due to the high contamination rates that resulted from their use. This is in contrast to the results reported for midstream urine samples collected from cattle and inoculated into the same media (Hellstrom, 1978) and reflects the difficulties experienced in obtaining uncontaminated urine from small animals compared with large domestic species.

No isolations were made from urine filtrates. Nervig and Ellinghausen (1978) found that $2.2 \times 10^5$ organisms/ml of urine were necessary before organisms could be cultured from filtrates and Turner (1970) also noted that large numbers of leptospires had to be present in urine samples for isolation after filtration to be successful. Dark-field examination of urine samples from possums in the present study failed to detect large numbers of leptospires in any urines examined. It is therefore likely that the number of leptospires that were present in the majority of urine samples were below the threshold necessary for the successful culture of filtrates. Leptospires were detected in a small number of uncentrifuged urine samples, however none were detected in samples that had been centrifuged. Similar observations were made by Doherty (1966) who examined urine samples from infected guinea pigs.

The relative efficiency of kidney culture, histological examination of kidneys, urine culture and dark-field microscopy of urine to demonstrate leptospiral infection in the present study was similar to that reported in other studies using similar techniques. Gordon-Smith *et al.* (1961) found 13% of Malayan rodents to be infected by cultural and serological methods, whereas only 4% were positive by histological examination of kidney sections and 1% by dark-field microscopy of urine. A survey of hedgehogs by Wolff (1965) found 26% to be positive by kidney culture whereas only 4.6% were positive by urine culture.

The dilution experiment supported the general findings of the cultural survey and provided quantitative estimates of the sensitivity of the techniques used. It was confirmed that media without 5FU and media with 200µg 5FU/ml were unsuitable for urine culture, however media containing 400µg 5FU/ml successfully prevented contamination of urine cultures and isolates were recovered from urine inocula containing as few as 55 leptospires.

There was a marked contrast in the sensitivity of direct culture and animal inoculation techniques for the isolation of leptospires from urine. The high MID and MLD for hamsters inoculated with urine containing leptospires illustrated that direct culture in media containing 400 µg 5FU/ml was a much more

sensitive method for isolating leptospires from urine.

The number of leptospires needed to infect hamsters was greater for urinary leptospires compared with leptospires maintained in media. This has been noted by other workers also. Nervig and Ellinghausen (1978) found that the $LD_{50}$ and $ID_{50}$ for hamsters infected with serovar *grippotyphosa* exposed to swine urine was 5926 and 696 respectively, while for organisms maintained in liquid media, the values were 87 and 2 respectively. These workers suggested that exposure to urine decreased the inherent infectivity and virulence of leptospires. In their study, urine was added to a media-adapted culture to demonstrate the toxic effect rather than using urine from a leptospiruric animal. This may have exaggerated the toxic effect of urine on leptospires and hence may have exaggerated the difference in sensitivity of culture inoculation compared with inoculation with urinary leptospires.

It has been shown that serovar *grippotyphosa* in kidney suspensions is virulent for gerbils and hamsters (Tripathy and Hanson, 1974). Gerbils exposed to avirulent media-adapted *grippotyphosa* were re-exposed to urine from shedding animals and no deaths resulted. These workers concluded that the primary inoculation had protected the animals from the subsequent challenge, despite the fact that only two of five gerbils in the group had produced titres to the primary challenge, both of which were low. In light of the present work, an alternative explanation maybe that the leptospires from the urine were of too low an infectivity to have been able to establish infection.

Increasing the dilution of possum urine in the dilution experiment did not increase the recovery of leptospires. This indicated that the decrease in number of leptospires in inocula from higher urine dilutions had more effect on number of isolations than any possible toxic effect of urine. This was also found in the field survey. Nervig and Ellinghausen (1978) reported a toxic effect on leptospires if urine was left for some time before culture. In the present work, urine samples were cultured immediately after collection, and therefore any such deleterious effect would have been avoided.

The dilution experiment showed that, when larger numbers of organisms were present in urine and kidney inocula ($10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions,) isolations were made from virtually all replicates (84 of 90 cultures in which no contamination occurred). The high repeatability of the cultural technique decreased as the number of leptospires in the inocula decreased, until, at the $10^{-5}$ and $10^{-6}$ dilutions, isolations within culture replicates were only sporadic. Therefore, the repeatability of the cultural technique is high when moderate or large numbers of leptospires are present in host tissue or fluids, but is low when few organisms are present.

The low repeatability of the cultural technique when few organisms are present in an inoculum is the probable explanation for the small degree of variability that occurred between the two types of media used in the field survey. The difference in total number of isolations was not significantly different for the two types of media, but isolations were made in six cases from one type of medium only. A variation in cultural efficiency was also noted at different times of culture after death. There was a decrease in number of isolations as time of culture after death increased, however two isolations were made from possums at the later time that were negative on initial culture. These two isolations indicated either cultural inefficiency at the time of initial culture or unilateral kidney infection. The author is not aware of any survey that has described unilateral kidney infection. There-fore it must be assumed that some degree of inefficiency exists in the recovery of leptospires from possum kidneys, despite the high prevalence of infection demonstrated with the cultural technique described.

Examination of comparative data from cultural regimes used by other workers reveals a similar, small degree of variation in cultural results. A comparison of Fletchers, Stuarts and EMJH media by Ryan (1978) showed that 94% of isolations of serovar *pomona* from pig kidneys were made in EMJH, 78% in Stuarts and 63% in Flet-chers. Despite the obvious superiority of EMJH, two isolations were made in Stuarts medium that were not made in EMJH. Roth *et al* (1961) reported that when a comparison of semisolid and solid media was made,

the greater majority of leptospiral isolations were made in semi-
solid medium, however a small number of isolations were made  on
solid medium that were not made in semisolid.  It is therefore
clear that any one cultural regime will not recover leptospires
from 100% of infected inocula.

Slightly fewer organisms were isolated in media containing
400 µg 5FU/ml compared with other media.  This difference was
not statistically significant, however it is possible that some
small degree of inhibition of leptospiral growth occurred in this
medium.  It has been found that inhibition can occur at levels
greater than 400 µg 5FU/ml when leptospires are  cultured in
Korthoff's medium (Hussaini and Ruby,1976).  Turner (1970) also
reported that 5FU possibly causes some inhibitionof leptospiral
growth.

The MID of *balcanica* and *pomona* for hamsters infected by the
IP route was 50 organisms and the respective MLD for both serovars
was 500 and 50 organisms.  The infectivity and virulence of
leptospiral serovars are not constant and may  vary considerably
between species.  Stalheim (1966  found a marked difference in the
MID and MLD of three strains of serovar *pomona* in hamsters.  Strains
Ohio and MLS had $MLD_{100}$ values of less than 150 and 50 organisms
respectively, whereas the MID for the Wickard strain was 1000
leptospires.  The MLD for *canicola* in hamsters was reported as
being two organisms (Ellinghausen, 1976).  Faine (1962) found
that the MID for mice infected with *australis* B was $3.8 \times 10^3$
leptospires.

The IP route was more effective than the PC route for
establishing infection in hamsters but once the hamsters were
infected there was no difference in the pathogenesis of the
disease.  The route of infection has been shown to affect the MID,
but not the pathogenesis of the disease, in other species (Stavitsky,
1945; Sullivan, 1970; Kiktenko *et al*,1976).

Individual variation in the response of hamsters to infection
was shown in some groups.  The occurrence of asymptomatic infection
in a small proportion of hamsters illustrated the need to culture
all surviving hamsters in experimental investigations, rather than

using death as the sole criterion of infection.

## SUMMARY AND CONCLUSIONS

1.  The use of the Coleworth Stomacher was shown to be an efficient method for the culture of kidneys.

2.  Kidney homogenate was the most suitable material for the isolation of leptospires. The number of isolations decreased with a delay in time of culture following death. Media containing 400 µg 5FU/ml was found to be unnecessary for the culture of kidneys and therefore the possibility of inhibition of growth of small numbers of organisms in this media was avoided.

3.  Histological examination of kidney sections, culture of filtered urine and dark-field microscopy of urine were inefficient techniques for the diagnosis of leptospiral infection in possums.

4.  A small degree of variation in culture efficiency occurred when culture results from different types of media were compared.

5.  The only media suitable for urine culture was that containing 400 µg 5FU/ml. A neat and 1:10 dilution of urine were used as inocula in future work.

6.  The repeatability of isolations made from culture of host tissue and fluids was high when high numbers of organisms were present in the inoculum, but it was low when low numbers were present.

7.  Serovar *balcanica* was pathogenic for hamsters, with a MID and MLD by the IP route of 50 and 500 organisms respectively.

8.  Leptospires in urine were less infective for hamsters compared with leptospires maintained in culture media.

9.  Hamsters were shown to be useful for the isolation of leptospires from contaminated material.

CHAPTER VI

CULTURAL AND SEROLOGICAL INVESTIGATIONS

OF PASTORAL POSSUM POPULATIONS.

INTRODUCTION

Agglutination tests can be regarded as "potentially serogroup-indicative only" (Turner, 1968). The discovery of a high prevalence of Hebdomadis serogroup titres in pastoral possum populations made the isolation and identification of the infecting leptospire a primary objective of the investigation of the epidemiology of leptospirosis in possums.

It has been well established that several different serovars may infect different animal species sharing a specific ecosystem (Roth et al, 1964; Clark et al, 1966; Schnurrenberger et al, 1970; Hanson, 1976). In such cases, it appears that each serovar circulates predominantly within one species of animal, which is referred to as the maintenance host for that serovar (Roth, 1961; Gordon-Smith et al, 1961). A criterion defining a maintenance host population that is applicable to field data is the ratio of prevalence of titres in the population to the bacteriological prevalence, as determined by culture. If the ratio of titre prevalence to culture prevalence of a particular serovar is approximately unity or below, then the species involved is designated a maintenance host. If the ratio is high, then the species is designated an accidental or secondary host for that serovar.

This criterion has been used to define the maintenance hosts for particular serovars present in particular ecosystems both in free-living and domestic animal populations (Gordon-Smith et al, 1961; Roth et al, 1963; Trainer et al, 1963; Braun et al, 1964; Turner, 1967; Fennestad and Borg-Petersen, 1972). The identification of the infecting serovar and the determination that the possum is a maintenance host for this serovar are described in the following chapter. The concept of a maintenance host, using a laboratory mouse experimental model is further investigated in Chapter XV.

MATERIALS AND METHODS

Cultural studies were undertaken on pastoral possum populations from three localities.

1.   White Rock Station, March 1976 : A visit to this farm in autumn resulted in 65 possums being captured by trapping over five nights.   A field laboratory was set up and all possums were cultured within three hours of death.   Serological observations from this sample were compared with those made from the serological survey carried out on the same property in August 1975.

2.   Awahanga, March 1976 : The second pastoral locality from which possums were taken for culture was Awahanga station, a 6000 ha. hill country farm in the Porangahau district.   Possums were present in moderate density and were taken by trapping and nigh-shooting with APDB personnel.   All cultures were made in a field laboratory within three hours of the possum's death.   This property had been subjected to heavy commercial poisoning and trapping the previous year.   A total of 55 possums were cultured.

3.   Palmerston North district, April 1976 : A smaller sample of 34 possums was taken from farmland within a radius of eight miles of Massey University.   Due to the low population density on this well-developed farmland, the possums in this sample were taken from randomly-selected shelter belts, haybarns and small pine plantations.

The serological and cultural techniques used have been described previously.   In addition to the five antigens used in the preliminary serological survey described in Chapter IV, all sera were tested against *balcanica* antigen.

All isolates were serially sub-cultured in liquid medium until sufficient density was achieved for serotyping against standard antisera.   Thes included antisera against two leptospires of the Hebdomadis serogroup, *viz.*, serovar *hardjo* strain NHI2/309 and serovar *balcanica*, strain T78.

Definitive identification of leptospiral serovars by cross-agglutination absorption tests is beyond the capacity of most laboratories and is therefore carried out at WHO Reference Laboratories. Antisera were prepared in rabbits against eight isolates, all of which had been serotyped as belonging to the Hebdomadis serogroup. Paired isolates and antisera were forwarded to Ms.Catherine Sulzer, WHO Reference Laboratory, Atlanta, U.S.A., for serovar identification. A bovine Hebdomadis serogroup isolate, recovered from the urine of a dairy cow on the Massey University No. 1 Dairy Farm, was also sent for identification.

The accuracy of the serological test as a method of estimating the prevalence of infection, *viz.* sensitivity and specificity, were calculated from combined data from the three localities sampled. The sensitivity of the MAT at arbitrary minimum serum dilutions was also calculated. The sensitivity is the probability of a diagnostic test correctly identifying as positive those animals that truly are positive. The specificity is the probability of identifying as negative those animals that truly are negative (Schwabe *et al*,1977). The methods for determining sensitivity and specificity are given in Appendix III.

## RESULTS

Serological results revealed a high prevalence of Hebdomadis serogroup titres in the pastoral possum populations sampled, as was the case in the initial serological survey. Titres against *hardjo* antigen were found in sera from 73 of a total of 154 possums sampled (47%) and titres against *balcanica* antigen were found in sera from 66 of the possums sampled (43%).

Titres against antigens from serogroups other than the Hebdomadis serogroup were found in only three possums (2%). One transitional-stage female had a titre of 1: 96 to *ballum* as well as titres of 1:384 to *hardjo* and 1:96 to *balcanica*. A *ballum* titre of 1:96 was also recorded in a mature male. A titre of 1:48 to *pomona* was recorded in a mature male with titres of 1:1536 to *hardjo* and 1:96 to *balcanica*.

Table 6.1 : Serological and cultural results for mature adult possums from all localities sampled.

| Location | Mature Males | | | Mature Females | | |
|---|---|---|---|---|---|---|
| | *hardjo* | *balcanica* | culture | *hardjo* | *balcanica* | culture |
| White Rock | 0 | 0 | + | 0 | 0 | − |
| | 1536[a] | 96 | + | 192 | 192 | + |
| | 3072 | 768 | + | 0 | 0 | − |
| | 384 | 24 | + | 1536 | 96 | + |
| | 384 | 96 | − | 0 | 0 | + |
| | 96 | 24 | + | 192 | 24 | + |
| | 1536 | 384 | + | 0 | 0 | − |
| | 0 | 0 | − | 768 | 96 | − |
| | 96 | 0 | + | 96 | 0 | + |
| | 1536 | 768 | − | 768 | 96 | + |
| | 1536 | 768 | + | 768 | 384 | + |
| | 1536 | 768 | − | | | |
| | 384 | 384 | − | | | |
| | 0 | 0 | − | | | |
| | 0 | 0 | + | | | |
| | 192 | 48 | + | | | |
| | 768 | 384 | + | | | |
| | 0 | 0 | + | | | |
| | 192 | 384 | + | | | |
| Awahanga | 192 | 0 | + | 96 | 24 | + |
| | 48 | 0 | + | 3072 | 384 | − |
| | 768 | 96 | + | 96 | 0 | − |
| | 3072 | 384 | + | 192 | 24 | + |
| | 0 | 0 | + | 768 | 192 | + |
| | 192 | 24 | + | 384 | 48 | + |
| | 384 | 96 | + | 1536 | 192 | − |
| | 384 | 24 | + | 0 | 0 | − |
| | 1536 | 384 | − | 0 | 0 | − |
| | 384 | 48 | − | 384 | 48 | − |
| | 1536 | 384 | + | 384 | 48 | + |
| | 768 | 96 | + | 3072 | 768 | − |
| | | | | 1536 | 96 | + |

[a] reciprocal of titres

Table 6.1 continued.

| Location | Mature Males | | | Mature Females | | |
|---|---|---|---|---|---|---|
| | *hardjo* | *balcanica* | culture. | *hardjo* | *balcanica* | culture |
| Palmerston North | 0 | 0 | − | 384 | 96 | + |
| | 6144 | 768 | + | 1536 | 384 | + |
| | 0 | 0 | − | 192 | 48 | − |
| | 768 | 192 | + | 768 | 48 | − |
| | 192 | 48 | + | 768 | 96 | + |
| | 96 | 24 | + | 768 | 192 | + |
| | 768 | 96 | + | 0 | 0 | + |
| | 0 | 0 | − | 384 | 48 | + |
| | | | | 0 | 0 | − |
| | | | | 1536 | 96 | + |

Table 6.2 : Serological and cultural results for transitional-stage adult possums from all localities sampled.

| Location | Transitional-stage males | | | Transitional-stage females | | |
|---|---|---|---|---|---|---|
| | *hardjo* | *balcanica* | culture | *hardjo* | *balcanica* | culture |
| White Rock | 96[a] | 0 | + | 6144 | 1536 | - |
| | 384 | 192 | - | 3072 | 768 | - |
| | 6144 | 768 | - | 384 | 96 | + |
| | 0 | 0 | - | 0 | 0 | - |
| | 0 | 0 | - | 0 | 0 | - |
| | 0 | 0 | - | 0 | 0 | - |
| | 768 | 384 | + | 0 | 0 | - |
| | 0 | 0 | - | 0 | 0 | - |
| | 0 | 0 | - | 0 | 0 | - |
| | 0 | 0 | - | 0 | 0 | - |
| | | | | 0 | 0 | - |
| Awahanga | 0 | 0 | - | 0 | 0 | - |
| | 192 | 48 | + | 0 | 0 | - |
| | 6144 | 384 | + | 0 | 0 | - |
| | 96 | 24 | + | 1536 | 192 | - |
| | | | | 12288 | 768 | + |
| Palmerston North | 0 | 0 | - | 0 | 0 | - |
| | 768 | 96 | - | - | - | - |
| | 384 | 48 | + | 384 | 192 | + |
| | 0 | 0 | - | 384 | 192 | - |
| | 0 | 0 | - | 0 | 0 | - |

[a] reciprocal of titre.

There was a marked difference in the age-specific serological prevalences for all samples (Tables 6.3, 6.4, and 6.5). The pattern observed was very similar to that observed in the initial serological survey of pastoral possums. The highest prevalence in each sample was found in the mature adult age-groups. Males and females in the transitional-stage adult age-groups had lower prevalences and only one titre was recorded in juveniles, that of 1:24 against *hardjo* antigen in a juvenile male weighing 1025 grams.

Leptospiral isolates were cultured from a total of 57 possums (37%). The sex-specific and age-specific bacteriological prevalences for possums from different localities are given in Tables 6.3, 6.4 and 6.5. There was a marked difference in age-specific bacteriological prevalence for possums from each locality, as was the case for the age-specific serological prevalence. Most isolations were made from mature adults and no isolations were made from juveniles.

The small sex differences in serological prevalence, bacteriological prevalence and combined serological and bacteriological prevalences that occurred in mature adults from each locality were shown to be statistically non-significant (Table 6.6). (There were insufficient numbers for statistical analysis of sex differences in these parameters for transitional-stage adults from each locality.) Thus different sexes within age-groups were combined for each locality. An analysis of differences in prevalence of *hardjo* and *balcanica* titres, *hardjo* titres and number of isolations, and *balcanica* titres and number of isolations, for mature adults from each locality, demonstrated that these differences were non-significant (Table 6.6). Despite the lack of statistical significance, there was a slightly lower bacteriological than serological prevalence in these groups.

The small differences in prevalence of *hardjo* titres, *balcanica* titres and the number of isolates when samples from different localities were compared were also non-significant (Table 6.6). Therefore data from possums from the three localities sampled were combined (Table 6.7).

Table 6.3 : Serological and bacteriological prevalence by age and sex of serogroup Hebdomadis infection in pastoral possums from White Rock.

| Classification | No. | No. seropositive | | Culture | No. sero and or culture positive | | GMT of positive sera | |
|---|---|---|---|---|---|---|---|---|
| | | *hardjo*(%) | *balcanica*(%) | (%) | | (%) | *hardjo* | *balcanica* |
| Mature male | 19 | 14 (74) | 13 (68) | 13 (68) | 16 | (84) | 1:570 | 1:206 |
| Mature female | 11 | 7 (64) | 6 (55) | 7 (64) | 8 | (73) | 1:424 | 1:108 |
| Transitional-stage male | 10 | 4 (40) | 3 (30) | 2 (20) | 4 | (40) | NT | NT |
| Transitional-stage female | 11 | 3 (27) | 3 (27) | 1 ( 9) | 3 | (27) | NT | NT |
| Juvenile male | 6 | 0 | 0 | 0 | 0 | | 0 | 0 |
| Juvenile female | 8 | 0 | 0 | 0 | 0 | | 0 | 0 |
| Total | 65 | 28 (43) | 25 (38) | 23 (35) | 31 | (48) | | |

Table 6.4 : Serological and bacteriological prevalence by age and sex of serogroup Hebdomadis infection in
pastoral possums from Awahanga.

| Classification | No. | No. seropositive | | | | Culture (%) | | No. sero and/or culture positive (%) | | GMT of positive sera | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *hardjo* | (%) | *balcanica* | (%) | | | | | *hardjo* | *balcanica* |
| Mature male | 12 | 11 | (92) | 9 | (75) | 10 | (83) | 12 | (100) | 1:494 | 1:104 |
| Mature female | 13 | 11 | (85) | 10 | (77) | 6 | (46) | 10 | (77) | 1:560 | 1: 96 |
| Transitional-stage male | 4 | 3 | (75) | 3 | (75) | 3 | (75) | 3 | (75) | NT | NT |
| Transitional-stage female | 5 | 2 | (40) | 2 | (40) | 1 | (20) | 2 | (40) | NT | NT |
| Juvenile male | 10 | 1 | (10) | 0 | | 0 | | 1 | (10) | NT | 0 |
| Juvenile female | 11 | 0 | | 0 | | 0 | | 0 | | 0 | 0 |
| Total | 55 | 28 | (51) | 24 | (44) | 20 | (36) | 29 | (53) | | |

Table 6.5 : Serological and bacteriological prevalence by age and sex of serogroup Hebdomadis infection in pastoral possums from Palmerston North.

| Classification | No. | No. seropositive | | Culture | No. sero and/or | GMT of positive sera | |
|---|---|---|---|---|---|---|---|
| | | *hardjo* (%) | *balcanica*(%) | (%) | culture positive (%) | *hardjo* | *balcanica* |
| Mature male | 8 | 5 (63) | 5 (63) | 5 (63) | 5 (63) | 1:668 | 1:110 |
| Mature female | 10 | 8 (80) | 8 (80) | 7 (70) | 9 (90) | 1:546 | 1: 96 |
| Transitional-stage male | 5 | 2 (40) | 2 (40) | 1 (20) | 2 (40) | NT | NT |
| Transitional-stage female | 5 | 2 (40) | 2 (40) | 1 (20) | 2 (40) | NT | NT |
| Juvenile male | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| Juvenile female | 4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 34 | 17 (50) | 17 (50) | 14 (41) | 18 (53) | | |

Table 6.6 : Chi-squared analysis of differences in prevalence of leptospiral titres and bacteriological prevalence in mature adult possums from White Rock, Awahanga and Palmerston North.

| Analysis | Class | White Rock | | Awahanga | | Palmerston North | |
|---|---|---|---|---|---|---|---|
| | | $X_c^2$ | P | $X_c^2$ | P | $X_c^2$ | P |
| Difference in prevalence by sex | *hardjo* titres | 0.03 | > 0.75 | 0.09 | > 0.75 | 0.09 | > 0.75 |
| | *balcanica* titres | 0.15 | > 0.5 | 0.13 | > 0.5 | 0.09 | > 0.75 |
| | culture | 0.03 | > 0.75 | 2.28 | > 0.1 | 0.2 | > 0.9 |
| | serology+culture | 0.08 | > 0.75 | 1.33 | > 0.1 | 0.68 | > 0.25 |
| Difference in sero. and bact. prevalence (combined sexes) | *hardjo* and *balcanica* titre prevalence | 0.08 | > 0.75 | 0.54 | > 0.25 | 0 | |
| | *hardjo* titres and culture | 0.01 | > 0.9 | 2.70 | > 0.1 | 0.01 | > 0.9 |
| | *balcanica* titres and culture | 0.01 | > 0.9 | 0.38 | > 0.5 | 0.01 | > 0.9 |

| Analysis | Class | $X^2$ | P |
|---|---|---|---|
| Difference in prevalence by location | *hardjo* titres | 0.84 | >0.5 |
| | *balcanica* titres | 1.23 | >0.5 |
| | culture | 0.39 | >0.75 |
| | culture +serology | 0.38 | >0.75 |

Table 6.7 : Age and sex prevalence of Hebdomadis serogroup titres and isolations from pastoral possums from White Rock, Awahanga and Palmerston North.

| Classification | No. | No. seropositive | | | | Culture | | No. seropositive and/or culture positive | | GMT of positive sera | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | *hardjo* | (%) | *balcanica* | (%) | | (%) | | (%) | *hardjo* | *balcanica* |
| Mature male | 39 | 30 | (77) | 27 | (69) | 28 | (72) | 33 | (85) | 1:555 | 1:148 |
| Mature female | 34 | 26 | (76) | 24 | (71) | 20 | (59) | 27 | (79) | 1:543 | 1:99 |
| Transitional male | 19 | 9 | (47) | 8 | (42) | 6 | (32) | 9 | (47) | 1:564 | 1:136 |
| Transitional female | 21 | 7 | (33) | 7 | (33) | 3 | (14) | 7 | (33) | 1:1534 | 1:348 |
| Juvenile male | 18 | 1 | ( 6) | 0 | | 0 | | 1 | ( 6) | NT | 0 |
| Juvenile female | 23 | 0 | | 0 | | 0 | | 0 | | 0 | 0 |
| Total | 154 | 72 | (47) | 66 | (43) | 57 | (37) | 77 | (50) | | |

Chi-squared analysis of combined serological and culture data from all localities showed that there was no significant sex difference in prevalence of Hebdomadis serogroup titres and bacteriological prevalence within adult age-groups (Table 6.9). *Hardjo* titres were found in sera from 56 of the 73 mature adults sampled (77%) and this prevalence was very similar to that of 75% recorded for mature adults in the initial serological survey (Table 4.4). The prevalence of *balcanica* titres in the same group was 70% (Table 6.8). Isolations were made from 48 of 73 mature adults (66%) and this bacteriological prevalence was not significantly different from the serological prevalence of both *hardjo* and *balcanica* titres (Table 6.9). Thus there was a very close association between the serological prevalences of *hardjo* and *balcanica* titres and the bacteriological prevalence in this group of animals.

A similar situation was observed in the transitional-stage adult age-group. There were no statistically-significant sex differences in serological or bacteriological prevalences (Table 6.9) and the bacteriological prevalence was not significantly different from the serological prevalence of *hardjo* or *balcanica* titres. This group however had a much lower prevalence of *hardjo* and *balcanica* titres (40% and 38% respectively) than the mature adults and isolations were obtained from only 9 of 40 animals (23%). These differences in prevalence between transitional-stage adults and mature adults were highly significant (Table 6.9).

The bacteriological culture prevalence was slightly lower than the serological prevalence in both adult age-groups but this difference was not statistically significant. The failure to isolate leptospires from juvenile animals was consistent with the absence of titres in this age-group.

GMT's for positive sera were calculated for both sexes in the adult age-groups. The GMT's for mature adult males and females and transitional-stage adult males were all of the same order (Table 6.7). In comparison, the GMT for transitional-stage adult females was considerably higher. This situation was not observed in GMT's calculated for adult possums collected in the initial serological survey in August.

Table 6.8 : Age prevalence of serogroup Hebdomadis titres and isolations in pastoral possums.

| Classification | No. | No. seropositive | | | | Culture (%) | | No. sero and/or culture positive (%) | |
|---|---|---|---|---|---|---|---|---|---|
| | | *hardjo* (%) | | *balcanica* (%) | | | | | |
| Mature adults | 73 | 56 | (77) | 51 | (70) | 48 | (66) | 60 | (82) |
| Transitional-stage adults | 40 | 16 | (40) | 15 | (38) | 9 | (23) | 16 | (40) |
| Juveniles | 41 | 1 | ( 2) | 0 | | 0 | | 1 | ( 2) |

Table 6.9 : Chi-squared analysis of differences in prevalence of leptospiral titres and bacteriological prevalence in mature adult and transitional-stage adult possums.

| Analysis | Class | LM and LF | | TM and TF | |
|---|---|---|---|---|---|
| | | $X_c^2$ | P | $X_c^2$ | P |
| Difference in prevalence by sex | *hardjo* titres | 0.05 | >0.25 | 0.34 | >0.5 |
| | *balcanica* titres | 0.87 | >0.25 | 0.06 | >0.75 |
| | culture | 0.01 | >0.9 | 0.85 | >0.75 |
| | serology + culture | 0.08 | >0.75 | 0.34 | >0.5 |

| Analysis | Class | Mature Adults | | Trans-stage Adults | |
|---|---|---|---|---|---|
| Difference in serological and bacteriological prevalence (combined sexes) | *hardjo* and *balcanica* titre prevalence | 0.63 | >0.25 | 0.01 | >0.9 |
| | *hardjo* titres and culture | 1.65 | >0.1 | 2.1 | >0.1 |
| | *balcanica* titres and culture | 0.13 | >0.5 | 1.5 | >0.1 |

| Analysis | Class | Mature Adults and Transitional-stage adults | |
|---|---|---|---|
| | | $X_c^2$ | P |
| Difference in prevalence by age | *hardjo* titres | 13.77 | < 0.005 |
| | *balcanica* titres | 9.36 | < 0.005 |
| | culture | 18.32 | < 0.005 |
| | serology + culture | 19.84 | < 0.005 |

Figure 6.1. : Prevalence of *hardjo* and *balcanica* titres, GMT's, and bacteriological prevalences for mature adult and transitional-stage adult possums.
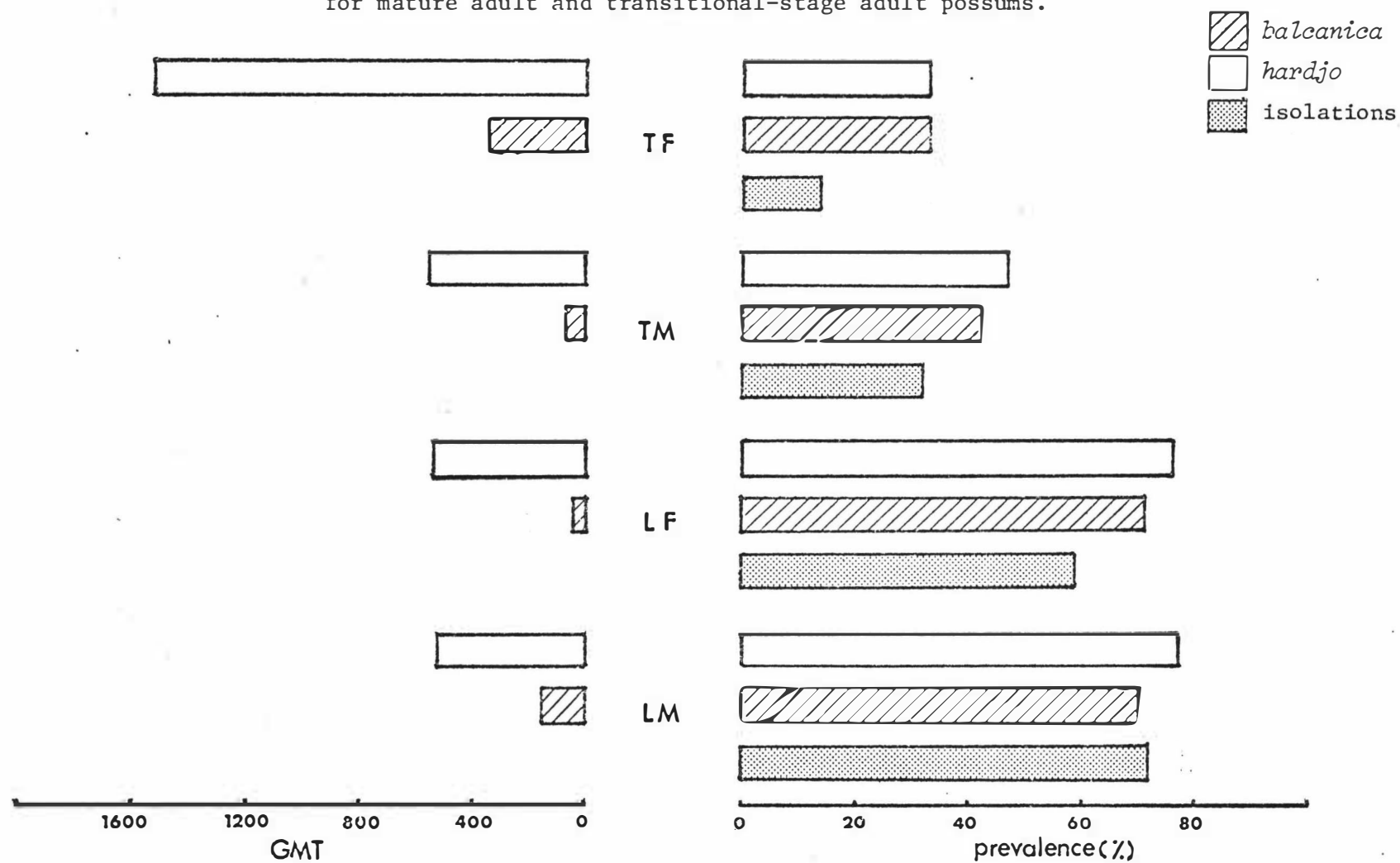
The 57 isolates recovered from kidney cultures were all serotyped as belonging to the Hebdomadis serogroup. No cross-reactions were observed with antisera produced against antigens from other serogroups. The eight isolates sent to the WHO Reference Laboratory were all identified as serovar *balcanica*. The bovine isolate was identified as serovar *hardjo*.

The finding of higher titres against *hardjo* the heterologous antigen, compared with the titres against *balcanica*, the homologous antigen, were consistent observations (Table 6.1 and 6.2). Although positively correlated to *hardjo* titres (r=0.62), *balcanica* titres were generally two to three serum dilutions lower. Thus the GMT's for *balcanica* titres in each sex and age-group were considerably lower than the GMT's for *hardjo* titres in the same groups (Table 6.7).

The frequency distribution of *hardjo* and *balcanica* titres for mature adults and transitional-stage adults is shown in Figures 6.2, 6.3, 6.4 and 6.5. In the mature adults, the frequency mode of *hardjo* titres was three serum dilutions higher than that for *balcanica* titres and the range of *hardjo* titres was from 1:24 to 1:6144. The range for *balcanica* titres was 1:24 to 1:768. A similar pattern was seen in the transitional-stage adults.

The occurrence of higher heterologous titres against *hardjo* than homologous titres against *balcanica* was a phenomenon observed only in possum sera. Of the 57 isolates serotyped against standard *hardjo* and *balcanica* antisera produced in rabbits, 48 gave higher titres against *balcanica* antisera than against *hardjo* antisera. Nine isolates gave equal titres against both antisera. (One of these nine isolates was identified as serovar *balcanica* by cross-agglutination absorption studies). This titre pattern in rabbits was confirmed when isolates that were sent to the WHO Reference Laboratory for serovar identification were tested against their own hyperimmune antisera produced in rabbits (Table 6.10). All gave higher titres against the homologous antisera. The *hardjo* isolate included as a control gave a higher titre against *hardjo* antisera than against *balcanica* antisera.
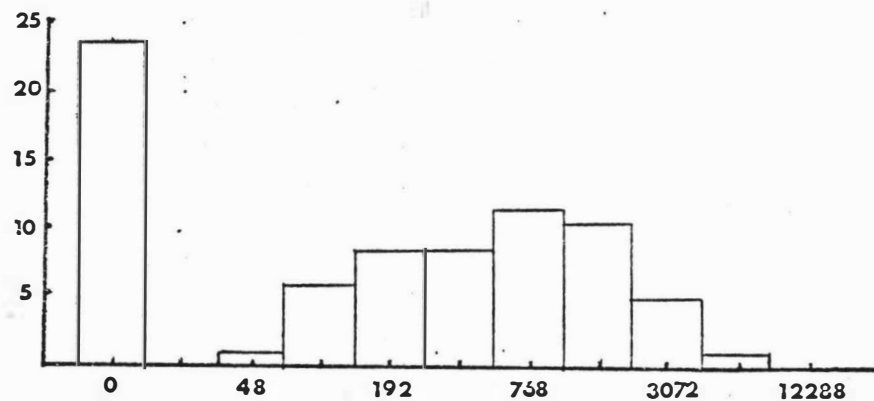
Figure 6.2. : Frequency distribution of *hardjo* titres in mature adult possums.
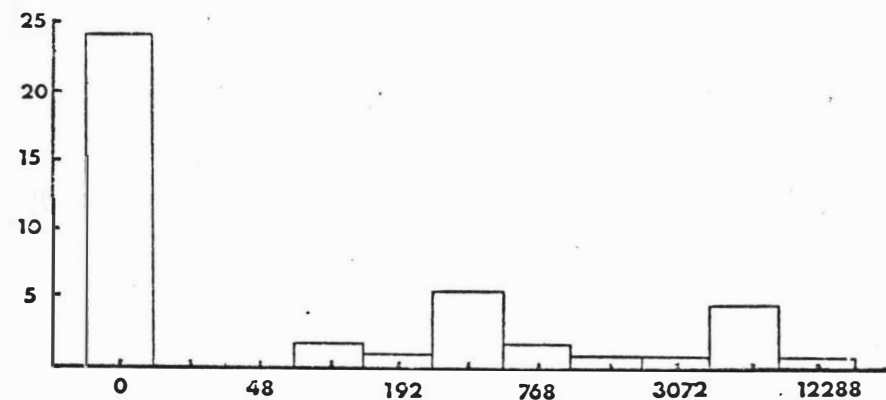


Figure 6.4. : Frequency distribution of *hardjo* titres in transitional-stage adult possums.
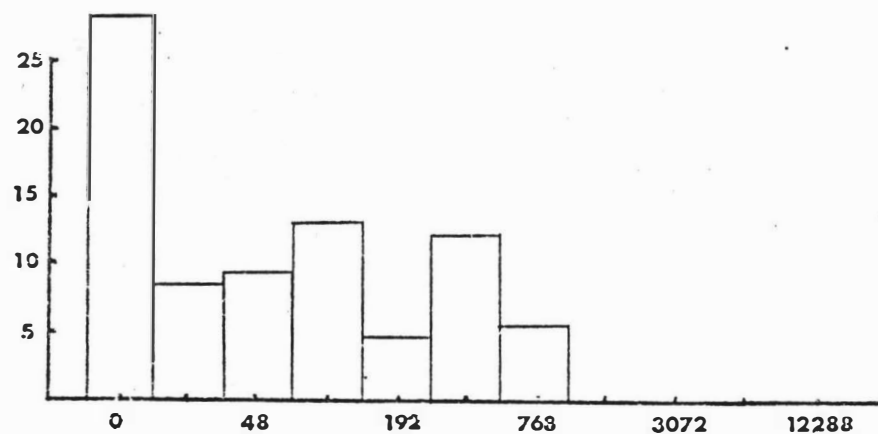


Figure 6.3. : Frequency distribution of *balcanica* titres in mature adult possums.
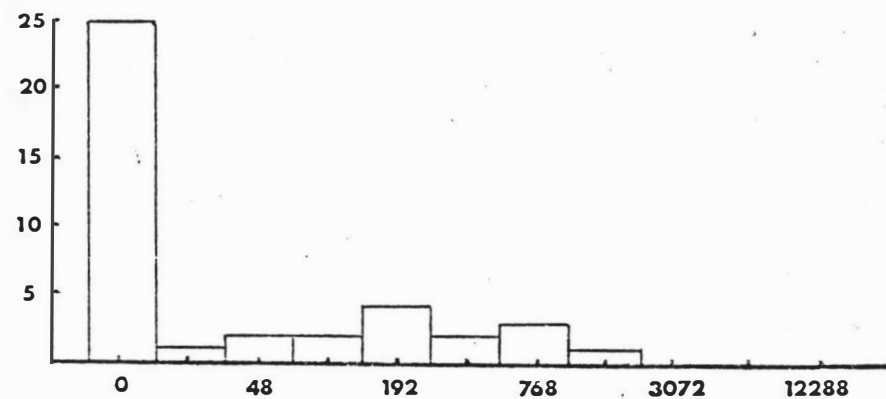


Figure 6.5. : Frequency distribution of *balcanica* titres in transitional-stage adult possums.

Table 6.10 : Serological reactions of isolates typed by cross-agglutination absorption tests as being serovar *balcanica*.

| Isolate | Possum Serum | | Serotyping [a] | | Hyperimmune antisera [b] | | Reference Lab. identification |
|---------|------|------|-------|-------|-------|-------|---------------------|
|         | H    | B    | H     | B     | H     | B     |                     |
| WR23    | 0    | 0    | 1536[c] | 12288 | 12288 | 98304 | *balcanica* |
| WR43    | 768  | 96   | 1536  | 6144  | 3072  | 49152 | *balcanica* |
| A 58    | 3072 | 768  | 3072  | 6144  | 3072  | 6144  | *balcanica* |
| A 60    | 3072 | 384  | 3072  | 3072  | 3072  | 12288 | *balcanica* |
| A 93    | 96   | 24   | 1536  | 6144  | 3072  | 12288 | *balcanica* |
| A 94    | 48   | 0    | 768   | 6144  | 3072  | 6144  | *balcanica* |
| EID     | 192  | 48   | 3072  | 3072  | 3072  | 6144  | *balcanica* |
| MÜ 1    | 1536 | 768  | 3072  | 6144  | 6144  | 49152 | *balcanica* |
| T 78    | 1536 | 384  | 1536  | 6144  | 1536  | 6144  | *balcanica* |
| 08/1    |      |      | 6144  | 1536  | 12288 | 3072  | *hardjo* |
| GMT of *balcanica* isolates | 1:588 | 1:190 | 1:1902 | 1:5612 | 1:3541 | 1:15,108 | |

[a] standard rabbit antisera, produced against : H = serovar *hardjo*, strain NHI 2/309
B = serovar *balcanica* , strain T78

[b] antisera produced against the isolate for cross-agglutination absorption procedures

[c] reciprocal of titre.

A comparison of the *hardjo* and *balcanica* titres of culture-positive mature adults showed that only 14 of 48 isolations (29%) were made from animals with *hardjo* titres ≤1:96, whereas 34 of 48 isolations (71%) were made from possums with *balcanica* titres ≤ 1:96 (Figures 6.6 and 6.7). This difference was highly significant ($X_c^2$ = 18.30, P <0.005). In contrast, 52% of culture-positive mature adults had *hardjo* titres in the 1:192 to 1:768 range, whereas only 29% of culture-positive animals had *balcanica* titres in this range. This difference was also statistically significant ($X_c^2$ = 4.25, P <0.05). The same trend was observed in transitional-stage adults (Figures 6.8 and 6.9). Numbers in this age-group were too small for statistical analysis.

The ability of the MAT to identify those adult possums that were culture-positive i.e. the sensitivity, is given in Table 6.11. When all mature adult sera were tested against *hardjo* antigen at a minimum serum dilution 1:24, the sensitivity was 0.90 and therefore false negatives occurred in 10% of animals. The sensitivity decreased as the minimum serum dilution increased until, when only *hardjo* titres of higher than 1:192 were considered to be test-positive, the sensitivity was 0.58. When mature adult sera were tested against *balcanica* antigen at a minimum serum dilution of 1:24, the sensitivity was 0.79 and therefore false negatives occurred in 21% of animals. As with *hardjo* titres, the sensitivity of *balcanica* titres decreased as the minimum serum dilution at which titres were read increased. Sensitivities were higher for transitional-stage adults than for mature adults.

The ability of the MAT to identify as negative those animals that were culture negative, the specificity, was lower than the sensitivity (Table 6.12). Therefore the MAT yielded a considerable number of false positives. Specificities were higher for the transitional-stage adults,for both *hardjo* and *balcanica* titres, than for mature adults.

The serological data for the samples taken from White Rock in August 1975, and March, 1976 are given in Table 6.13. Serological prevalences of *hardjo* titres for all sex and age-groups were lower

Figure 6.6. : Frequency of isolations from mature adult possums with different *hardjo* titres.

Figure 6.8. : Frequency of isolations from transitional-stage adult possums with different *hardjo* titres.

Figure 6.7. : Frequency of isolations from mature adult possums with different *balcanica* titres.

Figure 6.9. : Frequency of isolations from transitional-stage adult possums with different *balcanica* titres.

Table 6.11 : Sensitivity of the prevalence of *hardjo* and
*balcanica* titres relative to bacteriological
prevalence.

| Age-group | Serovar | Sensitivity of titre prevalence | | |
|---|---|---|---|---|
| | | titres >10 | titres >100 | titres >200 |
| Mature adults | *hardjo* | 0.90 (0.1)* | 0.75 (0.25) | 0.58 (0.42) |
| | *balcanica* | 0.79 (0.21) | 0.29 (0.71) | 0.23 (0.77) |
| Trans-stage adults | *hardjo* | 1.00 (0) | 0.78 (0.22) | 0.67 (0.33) |
| | *balcanica* | 0.89 (0.11) | 0.78 (0.22) | 0.33 (0.77) |

* false negative rate

Table 6.12 : Specificity of the prevalence of *hardjo* and
*balcanica* titres relative to bacteriological
prevalence.

| Age-group | Serovar | Specificity of titre prevalence | | |
|---|---|---|---|---|
| | | titres >10 | titres >100 | titres >200 |
| Mature adults | *hardjo* | 0.6 (0.4)* | 0.48 (0.52) | 0.52 (0.48) |
| | *balcanica* | 0.55(0.45) | 0.72 (0.28) | 0.36 (0.74) |
| Trans-stage adults | *hardjo* | 0.75(0.25) | 0.77 (0.23) | 0.77 (0.23) |
| | *balcanica* | 0.77(0.23) | 0.81 (0.19) | 0.90 (0.10) |

* false positive rate.

for the March sample. The GMT for positive sera was 1:615 for this sample, compared with the GMT of 1:740 for animals collected in August. The overall prevalence of *hardjo* titres in August was 65%, whereas that in March was 43%.


DISCUSSION

The serological observations made in the present survey were very similar to those made in the initial serological survey of pastoral possum populations. Hebdomadis serogroup titres were present in a high proportion of adult animals, there was no statistically-significant sex difference in serological prevalences and very few titres were found against antigens from other serogroups. These serological observations were discussed in Chapter IV.

An important observation in this survey was the consistent nature of age-specific serological prevalences in samples from geographically widely-separated localities. Statistical analysis demonstrated that the small differences that occurred were non-significant and therefore it would appear that there is very little variation in the epidemiology of leptospirosis in pastoral possum populations of equivalent density in the southern half of the North Island. This situation may be different in other pastoral localities, as Brockie (1975) reported that 23 possums from Helensville in the northern part of the North Island and six possums from Rabbit Island, near Nelson in the northern part of the South Island, were serologically negative.

The age-specific prevalence of leptospiral isolations was very similar to the age-specific serological prevalence and this para-meter was consistent in the three localities sampled. Isolations were made in all cases from sexually mature adults. This pattern has been observed in several surveys of leptospirosis in wildlife in other countries (Ferris *et al*, 1961; Gordon-Smith *et al*, 1961c; Wolff and Bohlander, 1965; Shotts *et al*, 1975) and may be a feature of endemic host-adapted leptospiral infection in free living populations. The absence of infection in sexually-immature animals

Table 6.13 : Prevalence of *hardjo* titres, GMT's and adult;juvenile
ratio's for pastoral possum samples from White Rock.

| Classification | August 1975 | March 1976 |
|---|---|---|
| Mature males | 91% | 74% |
| Mature females | 90 | 64 |
| Trans-stage males | 76 | 40 |
| Trans-stage females | 70 | 27 |
| Juvenile males | 7 | 0 |
| Juvenile females | 0 | 0 |
| | | |
| Whole sample | 65 | 43 |
| GMT(adults) | 1 :740 | 1:615 |
| Adult:juveniles | 2.8:1 | 3.64:1 |

indicates that they are either insusceptible (natural or immune resistance) or the circumstances necessary for infection are not met until the animal is sexually mature. These possibilities are investigated in Chapter XIII.A marked difference in prevalence with age also demonstrates the importance of age-differentiation when surveying wild animals for the prevalence of leptospiral infection.

The GMT's for transitional-stage females were considerably higher than those of the transitional-stage male and the mature male and female age-groups. An explanation for this observation can be found by considering the reproductive cycle of the possum, as described in Chapter II. The female has a relatively well-defined mating season. The main activity occurs during March and April, although small numbers of females may come into oestrus throughout the year. Consequently, the transitional-stage female group was for the first time engaging in mating and the associated changes in social behaviour at the time of sampling. In contrast, the adult male population retains the potential for sexual activity throughout the year. Due to the extremely variable age at which juveniles mature, the transitional-stage males as a group may exercise their mating potential several months earlier than their female counterparts. Consequently, as a group, transitional-stage males at the time of sampling (March and April) were not mating for the first time.

The high GMT for serologically-positive animals in the transitional-stage female age-group, suggestive of recent infection, indicated that it is during the first mating season that infection becomes established. (The GMT's for other adult age-groups were much lower and were within a similar range to each other, suggesting that infection had occurred at an earlier time in these groups). This hypothesis is supported by the absence of infection in sexually-immature possums although these animals are fully susceptible to experimental infection. (See Chapter VII).

The GMT's of male and female transitional-stage adults that were collected in August in the initial serological survey did not show the marked difference that occurred in the same groups

collected in the survey in March and April. Titres of females which
had become infected in March and April of that year would have been
waning by the time of collection in August and therefore no differ-
ence between males and females was demonstrated.

The finding of endemic *balcanica* infection in possums in
New Zealand established this animal as a maintenance host for this
organism. The ratios of the prevalence of *hardjo* titres and the
prevalence of *balcanica* titres to culture prevalence were 1.17:1
and 1.06:1 respectively and hence the basic criteria of Roth (1961)
and Gordon-Smith *et al*(1961) for the identification of a maintenance
host were satisfied. Although only 14% of isolates were typed by
cross-agglutination absorption, the serological evidence presented
suggests that all possum isolates were serovar *balcanica*. This
assumption is validated by the investigations reported in
Chapter X.

The failure to isolate leptospires from animals with titres to
antigens from serogroups other than the Hebdomadis serogroup sugg-
ested that these infections did not result in a chronic carrier
state. Thus the possum did not appear to be a maintanance host
for *ballum* or *pomona*. The very low prevalence of titres to these
antigens also suggests that possums seldom become infected with
these organisms. This may be due to a relative insusceptibility
to infection with these serovars, or a lack of contact.

The finding of widespread *balcanica* infection in a free-
living population in New Zealand was an interesting development
in the world distribution of this serovar, as it had only prev-
iously been reported from Eastern Europe. The type strain, 1627
Burgas, was isolated from a man in Bulgaria in 1958 and identified
as a new serovar by Babudieri and Mateev (1961). Subsequent
investigations revealed *balcanica* infection in domestic animals in
the North Osetia region of the U.S.S.R.(Semenova *et al*,1965). A
survey of animals passing through the Mozdok and Ozdzhonikidze
meatworks produced three *balcanica* isolates from pig kidneys and
one from a cow's kidney. The number of animals surveyed or
prevalence of other serovars was not given. Matveeva *et al* (1977)
also reported the isolation of *balcanica* from pigs, in the Moldavian

Republic, U.S.S.R.  These authors also did not document the prevalence of infection, however it was very low as only 9% of pigs were bacteriologically positive and serovars *hebdomadis, pomona* and *tarassovi* were the most common isolates.  It is therefore unknown whether domestic stock are maintanance hosts for *balcanica* in Eastern Europe.  Surveys of wild animals in Eastern Europe have failed to detect a *balcanica* reservoir, (Parnas *et al* ,1961; Mateev and Manev, 1974; Matveeva *et al*,1977) and therefore it is possible that the serovar circulates within the domestic animal population without reinforcement from a free-living species.

The occurrence of *balcanica* infection in humans or domestic animals in New Zealand is unknown.  Most leptospiral infections in these species are diagnosed by serology alone and all Hebdomadis serogroup titres have been considered in the past to be due to infection with serovar *hardjo*.  In light of the recognised role of cattle as the maintenance host for *hardjo* (Lake, 1973; Hanson, 1976) it is probable that a large majority of Hebdomadis serogroup titres in this species are due to infection with this organism. It is possible however that a small number of Hebdomadis serogroup infections in cattle and humans in New Zealand are due to *balcanica*, as this serovar has been isolated from these hosts in Eastern Europe. Although wildlife serovars are not usually endemic in domestic stock, there have been many reports of infection due to the same serovar in both wildlife and domestic animals sharing an ecosystem (van der Hoeden and Shenberg, 1962; Blood *et al*,1963; Alexander *et al*,1963; Roth *et al*,1964; Hanson *et al*,1964; Martin *et al*, 1967; Fennestad and Borg-Petersen, 1972; Hanson,1976).

Until *balcanica* is isolated from domestic animals or man, the occurrence of inter-species transfer in New Zealand can only be a matter of speculation, however it has been noted by some investigators that when this does occur, the manifestations of the disease may be more severe in the accidental host than they are in the maintenance host (Babudieri,1958; Blood *et al*,1963; Roth *et al*,1963; Torten *et al*,1970).  Therefore, particular attention should be paid to severe or atypical Hebdomadis serogroup infections in man and animals.

The isolation of *balcanica* from possums in New Zealand stimulated investigations of possums in Australia and initial serological results from the state of Victoria revealed the presence of Hebdomadis titres in several possums (P.Durfee,pers.comm.). Isolation of the infecting serovar from Australian possums proved very difficult however and it was only after stressing a caged animals with corticosteroids for 14 days that a *balcanica* isolation was made from kidney homogenate (Durfee and Presidente, 1977). Gordon (1977) described a severe outbreak of mastitis in dairy cows in Victoria, Australia, and found that urine from leptospiruric cows was lethal for guinea pigs. The outbreak was attributed to serovar *hardjo*, but the isolates obtained were not typed by cross-agglutination absorption. It is generally accepted that *hardjo* has very low pathogenicity for laboratory animals and there are no accounts of deaths to infection with this serovar in mice, hamsters or guinea pigs. It is therefore possible that the mastitis outbreak described was due to another, closely related member of the Hebdomadis serogroup, such as *balcanica*. The discovery of *balcanica* infection in Australian possums made it probable that *balcanica* in New Zealand possums originated from infection present in animals introduced to this country in the nineteenth century.

The results of the present culture survey throw serious doubt on the reports of *hardjo* infection in possums in New Zealand (Brockie 1975; de Lisle *et al*, 1975). The Hebdomadis isolates recovered by these investigators were not subjected to cross-agglutination absorption tests for serovar identification. The fluorescent antibody used by de Lisle *et al* (1975) was probably not sensitive enough to differentiate between *hardjo* and *balcanica* and the two Hebdomadis isolates obtained by Brockie (1975) were only subjected to typing against standard antisera. It is probable that the leptospires isolated by these investigators were in fact *balcanica*.

The occurrence of higher titres to *hardjo* antigen than to *balcanica* was a consistent finding. Higher titres may occur against serologically heterologous but antigenically related strains, as well as against unrelated serologically heterologous strains (Turner, 1970). Paradoxical reactions belonging to the latter

group have been well documented (Alston and Broom,1958; Roth *et al*, 1963; Minette and Shaffer, 1968). Menges *et al* (1960) found that dogs experimentally infected with *pomona* gave paradoxical titres to *autummalis* antigen for 237 days following inoculation. Homologous titres to *pomona* had fallen to zero well before this time. The occurrence of paradoxical reactions against serologically heterologous but antigenically related strains is less well documented. Individual cases have been reported (Alston and Broom, 1958; Fennestad, 1963) but the author is unaware of any reports of the consistent occurrence of paradoxical reactions such as was observed in the present survey. In one of the few accounts of experimental infection with an Hebdomadis serogroup organism in which titres were monitored against heterologous serovars from the Hebdomadis serogroup, Ellis and Michna (1977 ) found that titres to serovars *hebdomadis* and *sejroe* in bovines were a half to two $\log_{10}$ dilutions lower than titres to *hardjo*, the infecting serovar. A similar situation was found by Fennestad (1963) who experimentally infected a group of calves with *sejroe*. This author found that, generally, titres to the homologous serovar were higher than those against other members of the Hebdomadis serogroup, however on some occasions a paradoxical titre was recorded against serovar *saxkoebing*. It is interesting to note that a paradoxical reaction to *hardjo* was restricted to possums. Paradoxical titres to *hardjo* were not found in rabbits, or other species infected with *balcanica* (see. ChapterIX). In these species, *hardjo* titres were one or two $\log_2$ dilutions lower than *balcanica* titres.

The immunological basis of paradoxical reactions remains unexplained. The consistent occurrence of these reactions in possums may in part be related to the immunogenicity of *balcanica*. Comparative studies on antigenic extracts of serovars from the Hebdomadis serogroup (Manev and Siromashkova,1970) showed that fewer lines resulted from double diffusion in agar of *balcanica* extract compared with *sejroe* extract. These authors regarded this finding to be evidence that *balcanica* contained fewer antigenic components than *sejroe*. These authors also quote Babudieri and Mateev (1961) as regarding *balcanica* to be "antigenically poor" compared with other Hebdomadis serogroup organisms. Manev and Yanakieva (1973) produced four

absorbed sera to facilitate the typing of Bulgarian isolates from the Hebdomadis serogroup. Monospecific *sejroe*, *saxkoebing*, and *mini* antisera were produced, however these workers were unable to produce a monospecific *balcanica* serum as the *balcanica* activity was absorbed out by a combination of the other serovars. Hence a composite absorbed serum with a characteristic reaction pattern had to be used in place of a monospecific *balcanica* serum. This inability to produce a monospecific serum was regarded as further evidence of the poor antigenicity of *balcanica*.

The necessity of reading titres at low serum dilutions was well demonstrated in the present survey. If positive *balcanica* titres were considered to be only those of 1:192 or greater, then 34 of 48 isolations in mature adults would have been made from sero-negative animals (71%). Thus a survey limited to serology and having a minimum serum dilutions of around 1:200, as is often the case, would have grossly under-estimated the bacteriological prevalence. The very low serological to culture prevalence ratios recorded in some surveys of free-living populations (Martin *et al*, 1967 ; Schnurrenberger *et al*,1970; Shotts *et al*,1975; Brockie, 1977) are undoubtedly due to the high minimum serum dilutions at which titres have been read in these cases. Therefore the criterion of Roth (1961) for the identification of a maintenance host (ratio of serological to culture prevalence) must be interpreted relative to the minimum serum dilution at which titres are recorded.

The high sensitivity of the MAT when titres were read at an initial serum dilution of 1:24 provided additional data supporting the reading of low titres. The sensitivity of the test decreased as the minimum serum dilution at which titres were read increased. The sensitivity of *hardjo* titres was higher than that of *balcanica* titres due to the consistent occurrence of paradoxical reactions.

The specificity of mature-adult titres was higher for low titres than for high titres. This indicated that possums were long-term carriers of *balcanica*, as a large proportion of animals with waning titres were still culturally positive. (Experimental infections to quantify the length of time for which titres and infection persisted in possums are reported in the following chapter.) This

long-term excretion explained why some isolations were obtained
from sero-negative animals. It was probable in these cases that
titres had declined to zero even though animals were still infected.
An interesting contrast to this situation in possums is provided
by the specificity of *pomona* titres relative to *pomona* infection
in pigs (Ryan, 1978). This author found that the specificity of
titres of 1:96 or lower was very low, but this was much higher in
pigs with high titres. This reflected the short shedding time
of *pomona* by pigs, regarded by Ryan (1978) to be about three months.
Hence the majority of pigs had eliminated the infection by the time
titres had declined to 1:96 or lower, and therefore the specificity
of titres at these levels was very low. This was the converse
situation to that occurring in possums.

The predictive value of a test is a variable test statistic as
it changes with the prevalence of the disease (Schwabe *et al*, 1977).
The predictive value of the MAT is high in possum populations where
there is a high prevalence of Hebdomadis serogroup titres and
therefore it is a good indicator of bacteriological prevalence
(Table 6.14). The predictive value was even higher if only low
titres were considered (1:24 to 1:96) and this reflected the
high prevalence of infection in possums with low titres, i.e. the
chronic carriers. Serology thus provided an easily-applicable
technique for an estimation of the prevalence of infected animals
in a pastoral possum population.

The prevalence of *hardjo* titres in the August 1975 sample taken
from White Rock was lower than that of the sample taken in March 1976.
This may have been due to a seasonal effect on prevalence of titres
associated with a changing exposure pattern of different age-groups
in the population, or else a true difference in the prevalence of
infection in the two samples. The population sampled in March 1976
was situated approximately 11 km from the population sampled in August
1975, but it occupied a similar pastoral environment. Seasonal
differences in the prevalence of leptospirosis in free-living
populations have been reported (Popp, 1951; Ferris *et al*, 1961
Blood *et al*, 1963), and yearly fluctuations are also known to
occur (Altava *et al*, 1956). Variations in prevalence also occur
between adjacent free-living populations (Blood *et al*, 1963;

Table 6.14: Probability of adult possums with titres to *hardjo* and *balcanica* being culture positive (predictive value of serological prevalence).

| Age-group | Predictive value of titre prevalence | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Hardjo* titres | | | | *Balcanica* titres | | | |
| | all titres | 24-96 | 192-768 | ⩾1536 | all titres | 24-96 | 192-768 | ⩾1536 |
| Mature adults | 0.79 | 0.86 | 0.83 | 0.62 | 0.71 | 0.77 | 0.64 | NT* |
| Trans-stage adults | 0.53 | 1.00 | 0.63 | 0.29 | 0.53 | 1.00 | 0.44 | NT |

* insufficient numbers.

Wolff, 1963, quoted by Turner, 1967). The difference in age-structure of the two populations that was indicated by the different adult : juvenile ratios for the two samples may also have contributed to the different seological prevalence of *hardjo* titres.


## SUMMARY AND CONCLUSIONS

1.   There was very little variation in the prevalence of leptospiral infection in pastoral possum populations of similar density from three localities in the southern half of the North Island.

2.   No isolations were made from sexually-immature possums.

3.   Evidence was presented to suggest that it is during the first mating season that infection becomes established.

4.   All isolates were considered to be serovar *balcanica* and the possum was established as a maintenance host for this organism.

5.   The present study stimulated investigations of possums in Australia and *balcanica* was recovered from a possum in the State of Victoria, hence establishing the probable origin of infection in possums in New Zealand.

6.   Results of the present study refuted earlier reports of *hardjo* infection in possums in New Zealand.

7.   A consistent paradoxical reaction to *hardjo* antigen was found in the sera from possums infected with *balcanica*.

8.   The accuracy of the serological test (sensitivity and specificity) was high and the predictive value demonstrated that serology was a good indicator of infection in pastoral possums.

9.   A consideration of the accuracy of the serological test demonstrated that titres should be read at as low a minimum serum dilution as possible in serological surveys.

10.  The sensitivity and specificity of the serological test was used to identify possums as probable long-term carriers of leptospires.

CHAPTER VII

EXPERIMENTAL INFECTION IN POSSUMS

INTRODUCTION

Field investigations of leptospirosis in free-living populations are usually restricted to cross-sectional sampling, as techniques involving the repeated capture of marked animals are very difficult to apply.  Cross-sectional sampling provides data on the prevalence of titres and infection in the population under study, but provides only inferential data on parameters such as the pathogenesis of the disease in individual animals, the duration of titres and the length of time for which an animal remains infected.  This information can be gained only from experimental investigations.  Such information is useful in extending the knowledge gained from field observations and forming hypotheses on the epidemiology of the disease.

The determination of a maintenance host for a serovar, discussed in Chapter VI in terms of the ratio of serological and bacteriological prevalence, also requires additional information that can be gained only from experimental infection.  Roth *et al* (1963) characterised a maintenance host for a particular serovar and a long term excretor of viable, virulent organisms.  Chernukcha *et al* (1974) suggested that leptospiral infection in a maintenance host was characterised by a high and sustained titre, whereas infection by the same serovar in an accidental host was characterised by a low maximum titre that rapidly declined.  These workers also observed that leptospiruria in a maintenance host was constant, of high intensity and long duration and occurred in the majority of animals infected.  If leptospiruria occurred in an accidental host, it was intermittent, only low numbers of organisms were present and the duration of shedding was usually very limited.  Chernukcha *et al* (1974) also found that the young of free-living animals that were not maintenance hosts for a particular serovar were more susceptible than adults to experimental infection.  Ellis and Michna (1977 ) included the presence of only mild histological changes in kidneys as being a characteristic of leptospiral infection in a maintenance host.  This could be considered an extension of the concept of

"biological equilibrium" that was observed by Babudieri (1958) to exist between a host and a "host-adapted" serovar. This worker observed that "host-adapted" leptospires exerted only a minimal pathological effect on the tubular epithelium of the kidney of the host.

The definition of a maintenance host is complicated by the fact that one host species can be a reservoir for more than one serovar and one serovar may have maintenance hosts of different species in different regions (Roth *et al*, 1963; Turner, 1967; Michna, 1970). In addition, two maintenance hosts for the same serovar may occur in the same region (see Chapter XII) . Therefore, the maintenance host(s) for a particular serovar must be determined in the region under study and infectivity experiments must be conducted using locally-isolated serovars. Chernukcha *et al* (1975) demonstrated an inability to infect experimentally a sub-species of field mice from one geographic region with a particular serovar while a sub-species from another geographic region was fully susceptible and filled all criteria of a maintenance host for this serovar.

This chapter describes a series of experimental infections of the possum designed to investigate its role as a host for several leptospiral serovars isolated in New Zealand. A detailed investigation of the pathogenesis of *balcanica* infection in possums is also presented. Particular attention was paid to the virulence of leptospires in the various inocula as there have been a variety of conflicting reports on the virulence of leptospires maintained in artificial media. Several earlier workers found that the virulence of an isolate for laboratory animals decreased with serial subculture (van Theil, 1948; van der Hoeden, 1954; Stalheim,1966 b)however this appears to be less of a problem with the advent of EMJH media (Ellinghausen and Painter, 1976).

## MATERIALS AND METHODS

Juvenile possums were used for experimental infections as field studies have demonstrated the absence of infection in this age-group. Animals caught in the field were weighed on entry into the isolation unit and were individually-housed in wire mesh cages that had been disinfected by soaking in 5% Medol. The cages measured 37 x 37 x 60cm

and had hinged lids for access.  The diet of caged possums consisted
of hydrated feed peas, vegetables and apples.  The diets of young
juveniles were supplemented with milk.

All experimental animals underwent a pre-inoculation screen for
evidence of present or past leptospiral infection.  Two blood samples
for serology were taken by cardiac puncture and urine was obtained
for darkfield microscopy at a minimum interval of 21 days.  Sera were
tested against serovars *hardjo, balcanica, ballum, copenhageni, pomona*
and  *tarassovi* at a minimum serum dilution of 1:12.  Possums with
titres of 1:12 or higher were not used.  To minimise the risk of
seronegative carriers, urine was cultured for retrospective examination.

The animals were anaesthetised in a specially-constructed stainless
steel chamber with a perspex lid.  This made only a minimum handling
of possums necessary.  Both halothane[a] and ether were found to be
suitable agents for the anaesthetising of possums.  When halothane
was used it was introduced under pressure by means of an anaesthetic
machine.  When ether was used, a  wad of cotton wool under a grill in
the bottom of the anaesthetic chamber was soaked with ether before the
possum was introduced.  Ether had the advantage of being less expensive
and did not require the use of an anaesthetic machine, therefore this
was the agent used in the majority of cases.

Experimental inoculations were conducted by either the IP or PC
route.  For PC inoculation, the anaesthetised possum was placed in
dorsal recumbency, the abdomen shaved and the skin abraided with a
scalpel blade until hyperaemic.  An adhesive paper ring template
(1.5cm diameter) was applied and 0.1 ml of culture placed in the
centre.  Anaesthesia was maintained until the inocula had dried.

The protocol for monitoring experimental infections varied for
each experiment.  Blood samples for serology and culture were obtained
by cardiac puncture.  The possum was placed in lateral recumbency and a
22 gauge by one inch needle attached to a five ml disposable syringe was
introduced into the heart via the left side of the thorax.  Blood was
cultured immediately on collection of the sample.  Voided urine was

a  Imperial Chemical Industries Ltd., Cheshire, Great Britain.

collected in a sterile container prior to anaesthesia and also
cultured immediately. For collection of urine, the possum was
removed from its cage and held by the tail on a stainless steel
bench. The animal would become apprehensive due to its inability
to grip the smooth surface and this reaction was manifested by
micturition. (Urine could not be obtained by bladder puncture as
most animals urinated during the induction phase of anaesthesia).

Serological and cultural procedures have been described in
Chapter III. All experimental infections in possums were carried
out in parallel with hamster inoculations so as to determine the
infectivity and virulence of the inocula for hamsters. Hamster
inoculations by the IP and PC route have been described previously.

## Experiment 1 : Challenge of possums by the percutaneous route

This initial experiment was designed to find an infective dose
of serovar *balcanica* for possums. Possums were collected during
October and November, 1975. Of a total of 34 animals subjected to
the pre-inoculation screen, seven were rejected due to the presence of
low Hebdomadis serogroup titres. The possums were inoculated with
varying doses of *balcanica* by the PC route (Table 7.1.). Three possums
were maintained as controls. PC inoculation was chosen as several
investigators have suggested that leptospirosis can be transmitted via
this route (Alston and Broom, 1958; Roth, 1970). Scratches through to
the subcutis and other minor skin abrasions were often found in possums
and it was considered that these could facilitate any percutaneous
transmission under field conditions.

The *balcanica* culture used in this experiment had been subcultured
four times in EMJH media since isolation. The undiluted culture
contained $2 \times 10^8$ organisms/ml. Blood and urine samples were taken
from each possum every three days for two weeks and again at three weeks.
All urine and blood samples were cultured and urine was examined by
dark field microscopy. One kidney from each animal was cultured at
the termination of the experiment. Urine was tested for pH and the
presence of haemoglobin and protein using urinalysis and reagent strips[b].

[b]Bili-Labstix, Ames Company, Division of Miles Laboratories
Australia Pty Ltd. Melbourne, Australia.

An equal number of hamsters were inoculated with the same inocula that the possums received.

## Experiment 2 : Challenge of possums by the intra-peritoneal route

This experiment was regarded as a pilot experiment. Five juveniles captured at Awahanga in March 1976 were inoculated by the IP route. One possums (E2.3) was found in the pre-inoculation screen to have a titre of 1:96 to *hardjo* and 1:48 to *balcanica*. Three animals received $2 \times 10^6$ *balcanica* organisms and two received $2 \times 10^8$ organisms (Table 7.1). The culture used was the same strain as that used in the initial experiment and had been passaged five times since isolation. The possums were bled at two week intervals and one kidney from each possum was cultured at the termination of the experiment at ten weeks. The other kidney from each possum was retained for histological examination. Five pairs of hamsters, one pair for each inocula, were inoculated at the same time as the possums.

## Experiment 3 : Investigation of the virulence of *balcanica* organisms in kidney homogenate and artificial media

Two possums were each inoculated IP with 0.5 ml of a kidney homogenate from a moribund, *balcanica*-infected hamster. Two other possums were inoculated with 0.5 ml of a *balcanica* culture containing $5 \times 10^6$ organisms/ml. This isolate had been subcultured twice in liquid EMJH media since its isolation in semisolid medium six weeks previously. Each of two hamsters was inoculated with the same inocula that each possum received.

The possums were bled at 10 and 21 days after inoculation and kidneys and urine were cultured at the termination of the experiment at 21 days post-inoculation (p.i.).

## Experiment 4 : Pathogenesis of *balcanica* infection in possums

The pathogenesis of experimental *balcanica* infection in possums was monitored for a period of 13 months. This experiment also provided controlled conditions under which different methods for

determining leptospiral infection could be compared. Eight
juveniles, collected from the Woodville and Dannevirke districts
(Table 7.1) were inoculated with 5 x $10^6$ organisms by the IP route.
The isolate used had been subcultured twice in liquid media since
primary isolation. Two other juveniles were maintained as negative
controls. Six hamsters were each infected with a similar inoculation
to that which the possums received.

The possums were observed for clinical symptoms twice daily for
15 days following post inoculation. The leptospiraemic phase of
infection was monitored by serial bleeding at three day intervals
for the first 15 days p.i. These blood samples were cultured
according to standard techniques and 0.5 ml of each sample was
also inoculated into two hamsters. Blood samples collected at
days 21 and 28 p.i. were subjected to the same procedures. The
haemoglobin concentration and packed cell volume of blood samples,
taken over the first 28 days p.i., were monitored by standard
cyanmethaemoglobin and microhaematocrit techniques.

All blood samples were tested for the serological response to
infection. After the first 28 days p.i., blood samples were
collected at 30-day intervals until the experiment was terminated
at 390 days p.i.

The leptospiruric phase of infection was monitored by dark-field
microscopy of urine samples, urine culture and hamster inoculation.
Each of two hamsters were inoculated with 0.5 ml of urine at the
time of collection. In addition to the above procedures, all
urine samples were tested against *balcanica* and *hardjo* antigen in
the MAT, at a minimum urine dilution of 1:6. Urine samples for this
procecure were centrifuged before testing to remove particulate
matter. The presence of haemoglobin, protein and sugar in urine
was monitored using urinalysis reagent strips .

Both kidneys from each possum were cultured at the termination
of the experiment. A portion of one kidney from each animal was
taken for histological examination before cultural procedures were
carried out.

Experiment 5 : Challenge of possums with serovar *hardjo*

A total of ten juveniles were challenged with *hardjo* organisms
from several sources (Table 7.1). The *hardjo* isolate obtained from a
human blood sample had been maintained in liquid media for approximately
two years. The bovine isolate had been subcultured four times in
liquid media since primary isolation from urine. The dark-field
positive urine sample used for possums E5.9 and E5.10 was obtained
from a cow from a herd that had endemic *hardjo* infection. A lepto-
spire isolated from the urine of this cow was subsequently typed as
being serovar *hardjo*. These experiments were of three weeks duration,
with bleeding for serological examination and urine collection for
dark-field microscopy and culture being conducted at weekly intervals.
Kidneys from each possum were cultured at termination of each
experiment. The infectivity and virulence of the *hardjo* inocula for
hamsters was also monitored.

Experiment 6 : Challenge of possums with leptospires of the Ballum
Serogroup.

The detection of serogroup Ballum titres in a small percentage of
possums collected during field studies stimulated an investigation of
experimental infection with organisms of this serogroup (Table 7.1). Two
possums were each inoculated IP with 0.5 ml of a Ballum serogroup culture
that contained $5 \times 10^7$ organisms/ml. This isolate had been obtained
from a ship rat and had been subcultured twice in liquid media since
primary isolation. Two other possums were inoculated with 0.5 ml of
a serovar *ballum* culture containing $2 \times 10^8$ organisms/ml. This was a
fourth-subculture isolate that was subsequently typed by cross-agglut-
ination absorption. This experiment was conducted as for Experiment 5.

RESULTS

Serological and cultural procedures failed to provide any evidence
of leptospiral infection in juvenile possums inoculated with *balcanica*
by the PC route in Experiment 1. The culture used to inoculate the
possums was shown to be of high infectivity and virulence for hamsters.
All hamsters receiving $\geqslant 10^3$ organisms died between days 12 and 17 p.i.
One hamster inoculated with $10^2$ organisms also died (day 18 p.i.) and
one surviving hamster in this group was culture positive at day 21 p.i.

Table 7.1 : Juvenile Possums by Sex, Weight and Inoculum Received.

| Expt No. | Possum No. | Sex | Weight (g) | Inoculum | | | |
|---|---|---|---|---|---|---|---|
| | | | | serovar | origin | route of inoc. | No.organisms |
| E1 | 1 | F | 1025 | *balcanica* | possum | PC | $10^8$ |
| | 2 | F | 950 | " | kidney culture | " | " |
| | 3 | M | 925 | " | " | " | $10^6$ |
| | 4 | M | 950 | " | " | " | " |
| | 5 | M | 950 | " | | " | $10^4$ |
| | 6 | F | 725 | " | " | " | " |
| | 7 | M | 1300 | " | " | " | " |
| | 8 | F | 1150 | " | " | " | " |
| | 9 | M | 850 | " | " | " | " |
| | 10 | F | 1025 | " | " | " | $10^3$ |
| | 11 | M | 950 | " | " | " | " |
| | 12 | M | 900 | " | " | " | " |
| | 13 | M | 1025 | " | " | " | " |
| | 14 | F | 1250 | " | " | " | " |
| | 15 | F | 875 | " | " | " | $10^2$ |
| | 16 | M | 1250 | " | " | " | " |
| | 17 | F | 875 | " | " | " | " |
| | 18 | F | 825 | " | " | " | " |
| | 19 | M | 850 | " | " | " | " |
| | 20 | M | 800 | " | " | " | $10^1$ |
| | 21 | M | 900 | " | " | " | " |
| | 22 | F | 925 | " | " | " | " |
| | 23 | F | 975 | " | " | " | " |
| | 24 | M | 1050 | " | " | " | " |
| | 25 | F | 1125 | " | " | " | 0 |
| | 26 | M | 975 | " | " | " | " |
| | 27 | M | 1000 | " | " | " | " |

Table 7.1 cont.

| Expt. No. | Possum No. | Sex | Weight (g) | Inoculum | | | |
|---|---|---|---|---|---|---|---|
| | | | | serovar | origin | route of inoc. | No. organisms |
| E2 | 1 | M | 1900 | *balcanica* | possum kidney culture | IP | $10^5$ |
| | 2 | F | 1725 | " | | " | " |
| | 3 | M | 650 | " | " | " | " |
| | 4 | F | 1575 | " | " | " | $10^8$ |
| | 5 | F | 1500 | " | " | " | " |
| E3 | 1 | M | 1150 | " | hamster kidney homog. | " | NT |
| | 2 | M | 1475 | " | | " | NT |
| | 3 | F | 1250 | " | possum kidney culture | " | $2.5 \times 10^6$ |
| | 4 | M | 1200 | " | | " | " |
| E4 | 1 | F | 1125 | " | possum kidney culture | " | $5 \times 10^6$ |
| | 2 | M | 1250 | " | | " | " |
| | 3 | M | 1100 | " | " | " | " |
| | 4 | M | 1725 | " | " | " | " |
| | 5 | M | 1400 | " | " | " | " |
| | 6 | F | 1000 | " | " | " | " |
| | 7 | F | 1075 | " | " | " | " |
| | 8 | M | 1300 | " | " | " | " |
| | 9 | M | 1225 | " | " | " | " |
| | 10 | F | 1125 | " | " | " | " |
| E5 | 1 | M | 1775 | *hardjo* | human blood culture | " | $10^5$ |
| | 2 | M | 850 | " | | " | " |
| | 3 | F | 1025 | " | " | " | $10^8$ |
| | 4 | F | 1800 | " | " | " | " |
| | 5 | M | 1125 | " | bovine urine culture,strain1 | " | $5 \times 10^7$ |
| | 6 | F | 1250 | " | " | " | " |
| | 7 | M | 1250 | " | "strain 2 | IM & IP | $2 \times 10^8$ |
| | 8 | F | 1200 | " | " | " | " |
| | 9 | M | 875 | " | bovine urine | " | NT |

Table 7.1 cont.

| Expt. No. | Possum No. | Sex | Weight (g) | Inoculum | | | |
|-----------|-----------|-----|-----------|---------|--------|--------|--------|
| | | | | serovar | origin | route of inoc. | No. organisms |
| E5 | 10 | F | 1525 | *hardjo* | bovine urine | IM & IP | NT |
| E6 | 1 | M | 1200 | Ballum* | ship rat kidney culture | IP | $2.5 \times 10^7$ |
| | 2 | F | 900 | | | " | " |
| | 3 | F | 2100 | *ballum* | mouse kidney culture | " | $10^8$ |
| | 4 | F | 1750 | " | | " | " |

* Ballum serogroup i.e. cross-agglutination absorption not performed on this isolate.

The pH of urine samples varied from 6.2 to 7.8 and the pH of serial urine samples from individual animals were also very variable (range 6.4 to 7.8). This precluded the use of pH measurements as a parameter of renal infection in later experiments. Protein was present in 32% of urine samples tested and showed an inconsistent pattern for individual animals. Haemoglobin was not detected in any urine samples. Two possums in Experiment 1 died from cardiac rupture following blood sampling.

Evidence of infection was found in four of five possums inoculated with *balcanica* by the IP route in Experiment 2. The serological response to infection of possums E2.1, E2.5 and E2.6 is shown in Figure 7.1. As was the case in field surveys, titres to *hardjo* antigen were consistently higher than titres to *balcanica* antigen. Peak titres occurred in sera collected on day 14 p.i. and the highest titre recorded was 1:24, 576 to *hardjo* antigen in possum E2.1. Serial serum samples from each possum showed that there was a large degree of individual variation in the level at which titres persisted. Titres declined rapidly in possum E2.1 and were present at only low levels at day 70 p.i. Titres were present in sera from possum E2.5 only on day 14 p.i. The most substained serological response to infection occurred in possum E2.6 with titres of 1:768 to *hardjo* and 1:48 to *balcanica* at day 70 p.i. The only titre against antigens from other serogroups was a titre of 1:24 to *pomora* which occurred at day 14 p.i. in possum E2.1.

Titres recorded in sera from possum E2.3 are also shown in Figure 1. Titres present before inoculation showed a gradual decline over the ten week duration of the experiment. There was no serological response to inoculation in this animal.

Possum E2.2 died from an acute gastro-enteritis possibly associated with overfeeding with greenfeed on day 17 p.i. A serum taken at day 14 p.i. had titres of 1:6144 to *hardjo* and 1:536 to *balcanica*. No kidney culture was made from this animal.

Serovar *balcanica* was recovered from kidney cultures at day 70 p.i. from possums E2.1, E2.5 and E2.6. Leptospires were not recovered from possum E2.3.
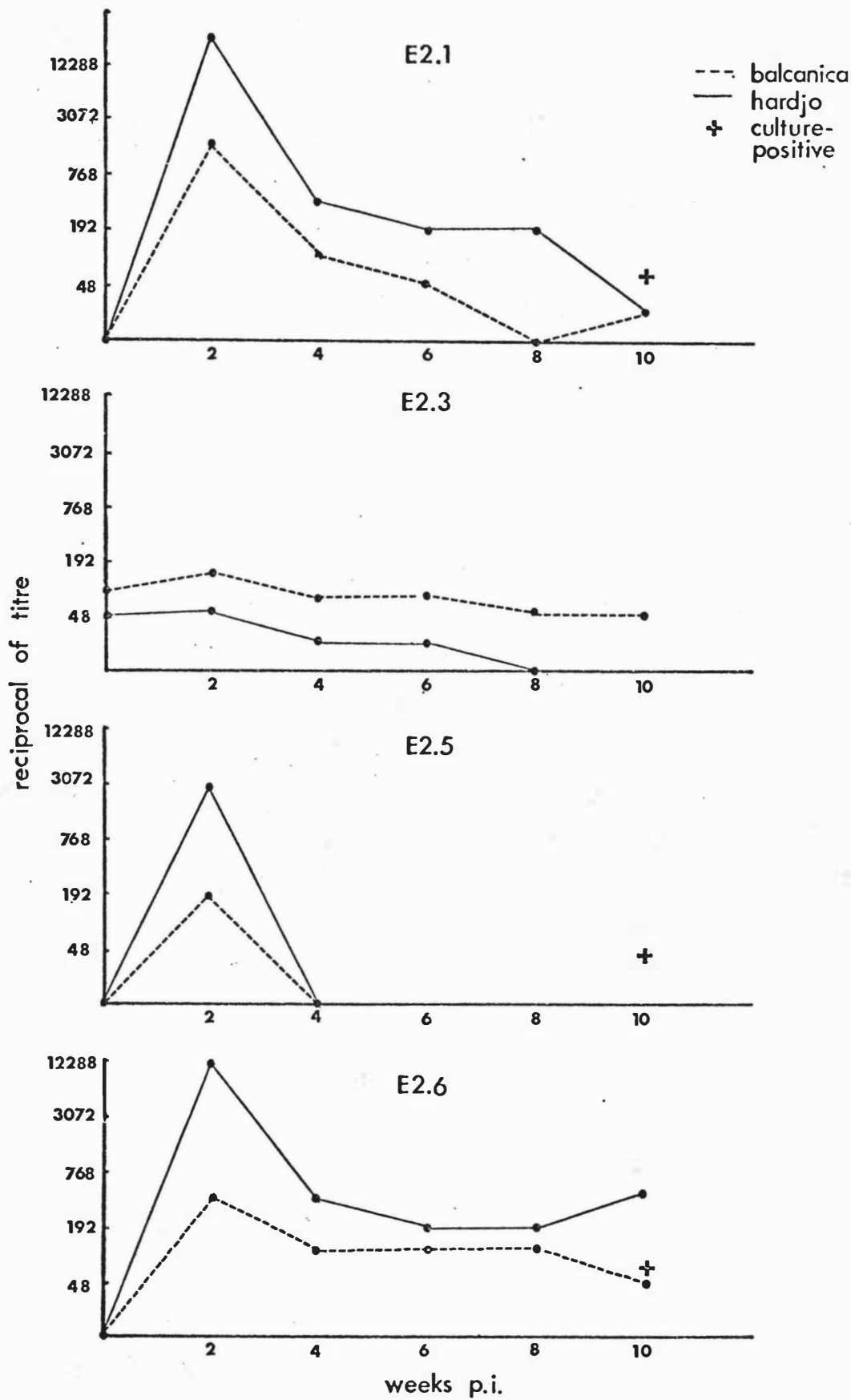
Figure 7.1. : *Hebdomadis* serogroup titres in possums experimentally infected with *balcanica* (Experiment 2).

The average weight-gain of the four animals surviving the experiment was 410 g. This was greater than would be expected under field conditions.

The mean death time of hamsters infected with the *balcanica* culture used in Experiment 2 was 14.2 days. Only one hamster survived the inoculum of $2 \times 10^6$ organisms, however this animal was culture-positive at 21 days p.i.

*Balcanica* organisms present in hamster kidney homogenate (Experiment 3) were not shown to be more virulent for possums than organisms cultured in artificial media. No clinical symptoms were apparent in infected animals and titres in possums inoculated with hamster homogenate were similar to those in possums infected with organisms cultured in media (Table 7.2). Possum E3.4 failed to develop a titre and this was the only animal in this group from which *balcanica* was not recovered at day 21 p.i. This possum had had a titre of 1:24 to *balcanica* at the first pre-inoculation screen 28 days before inoculation, however, this titre had dropped to zero at the second pre-inoculation screen seven days before inoculation.

The mean death time of hamsters infected with hamster kidney homogenate was 12 days. This was slightly shorter than the mean death time of 13.6 days recorded for hamsters infected with orgamisms cultured in media.

Experiment 4 provided detailed information on the pathogenesis of *balcanica* infection in experimentally-infected possums. Hebdomadis serogroup titres were first detected on day six p.i. and reached a peak in all possums between day 9 and 15 p.i. Maximum titres recorded were 1:12,288 to *hardjo* and 1:768 to *balcanica* in possum E4.6. Titres to both antigens fell rapidly from peak levels in most animals however the titres reached a plateau at day 60 p.i. and were subsequently maintained at almost constant levels for the remaining 11 months of the experiment. The serological responses of individual animals are shown in figures 7.2 to 7.7. Possum E4.1 died due to cardiac rupture following heart bleeding on day six p.i. and possum E4.3 died during anaesthesia on day 15 p.i. therefore data on these individuals is not presented. There was no

Table 7.2 : Hebdomadis serogroup titres in possums experimentally
infected with *balcanica* in Experiment 3.

| Possum No. | Titre at 10 days p.i. | | Titre at 21 days p.i. | | Culture (21 days p.i.) |
|---|---|---|---|---|---|
| | *balcanica* | *hardjo* | *balcanica* | *hardjo* | |
| E3.1 | 1:192 | 1:768 | 1:384 | 1:3072 | + |
| E3.2 | 1:48 | 1:192 | 1:192 | 1:1536 | + |
| E3.3 | 1:192 | 1:1536 | 1:768 | 1:3072 | + |
| E3.4 | 0 | 0 | 0 | 0 | − |

serological response to inoculation in possum E4.3.

There was some degree of variation in the serological responses
of individual animals. Peak titres varied from 1:12,288 to 1:1,536
to *hardjo* and from 1:768 to 1:192 to *balcanica*. Titres in possums
E4.4 and E4.6 showed a marked peak during the acute phase of
infection whereas such a peak was absent in possum E4.2. Fluct-
uations in titres of serial serum samples from individual animals
in the chronic stage of infection probably reflected a variation
in the precision of the MAT rather than real fluctuations in titres.
(The precision estimate gained from the repeat testing of 100 sera
in Chapter IV demonstrated that 45% of titres were the same and 86%
of titres were within $\pm$ one serum dilution.)

There was a general tendency in all animals for *hardjo* titres to
be more closely related to *balcanica* titres in the chronic stage
of infection compared with the acute stage. This tendency is
reflected in the GMT's of serial serum samples from all animals
(Table 7.3 and Figure 7.8) and is examined more closely in terms
of the antibody classes involved in Chapter IX. Despite this
tendency,*hardjo* titres were higher than or equal to *balcanica*
titres in all but one case.

No clinical symptoms were observed in infected possums. The
monitoring of haemoglobin and packed cell volume gave inconclusive
results as a blood loss anaemia, due to serial blood sampling,
occurred in both infected animals and controls. There was no
evidence of haemoglobinuria or glucosuria in any of the infected
animals.

The duration of leptospiraemia varied from 3 to 15 days (Table
7.4). In six out of seven infected animals, leptospires were
demonstrated in blood after the appearance of leptospiral
agglutinins.

Leptospiruria commenced between days 3 and 12 in different
animals (Figures 7.2 to 7.7). Darkfield microscopy demonstrated
that shedding was of maximum intensity in most animals between
days 28 and 60 p.i. Possum E4.2 was an exception in that large
numbers of organisms (more than $16^6$ leptospires/ml urine) were
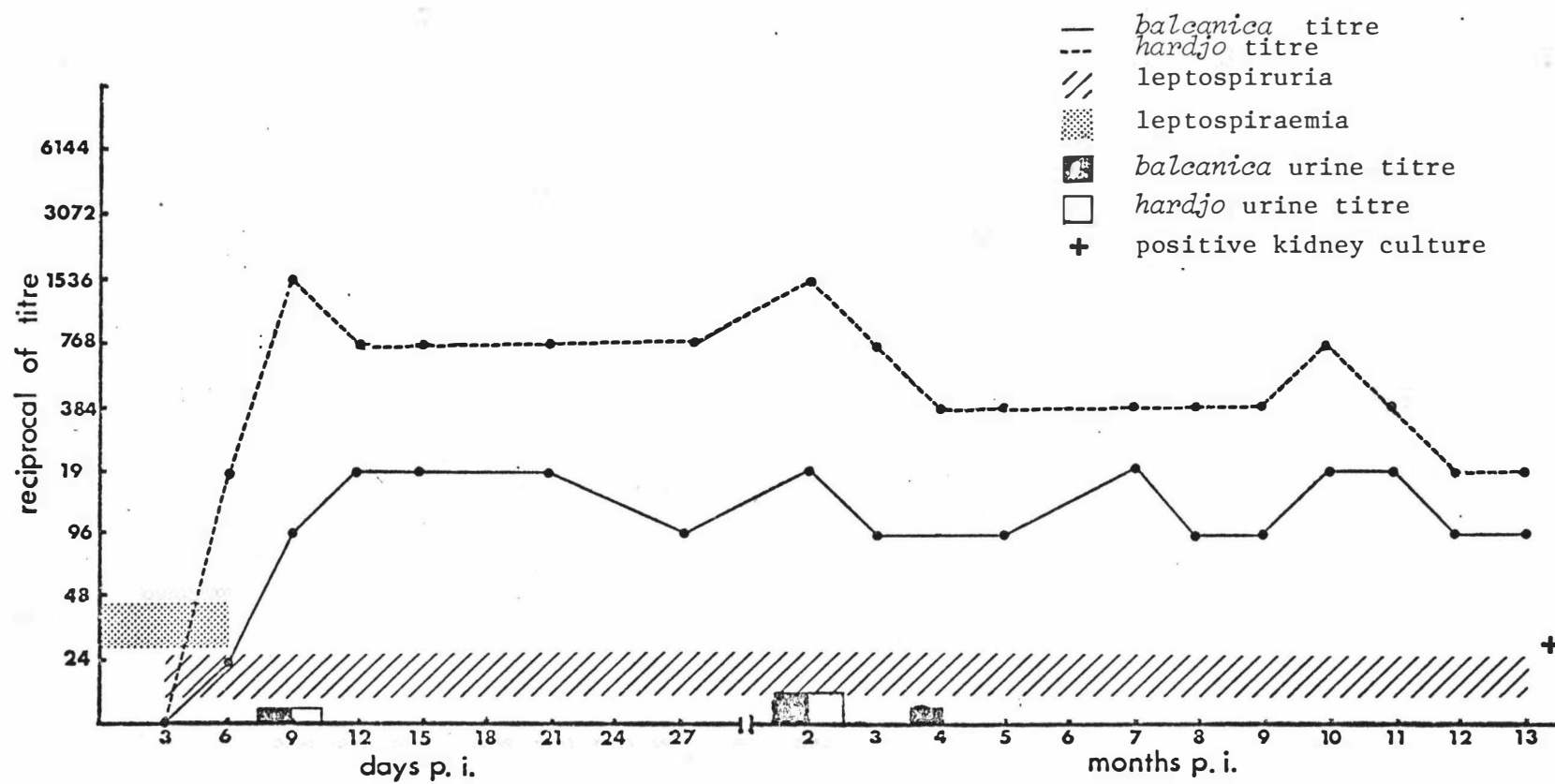
Figure 7.2. : Experimental infection with *balcanica* in possum E4.2

Figure 7.3. : Experimental infection with *balcanica* in possum E4.4.
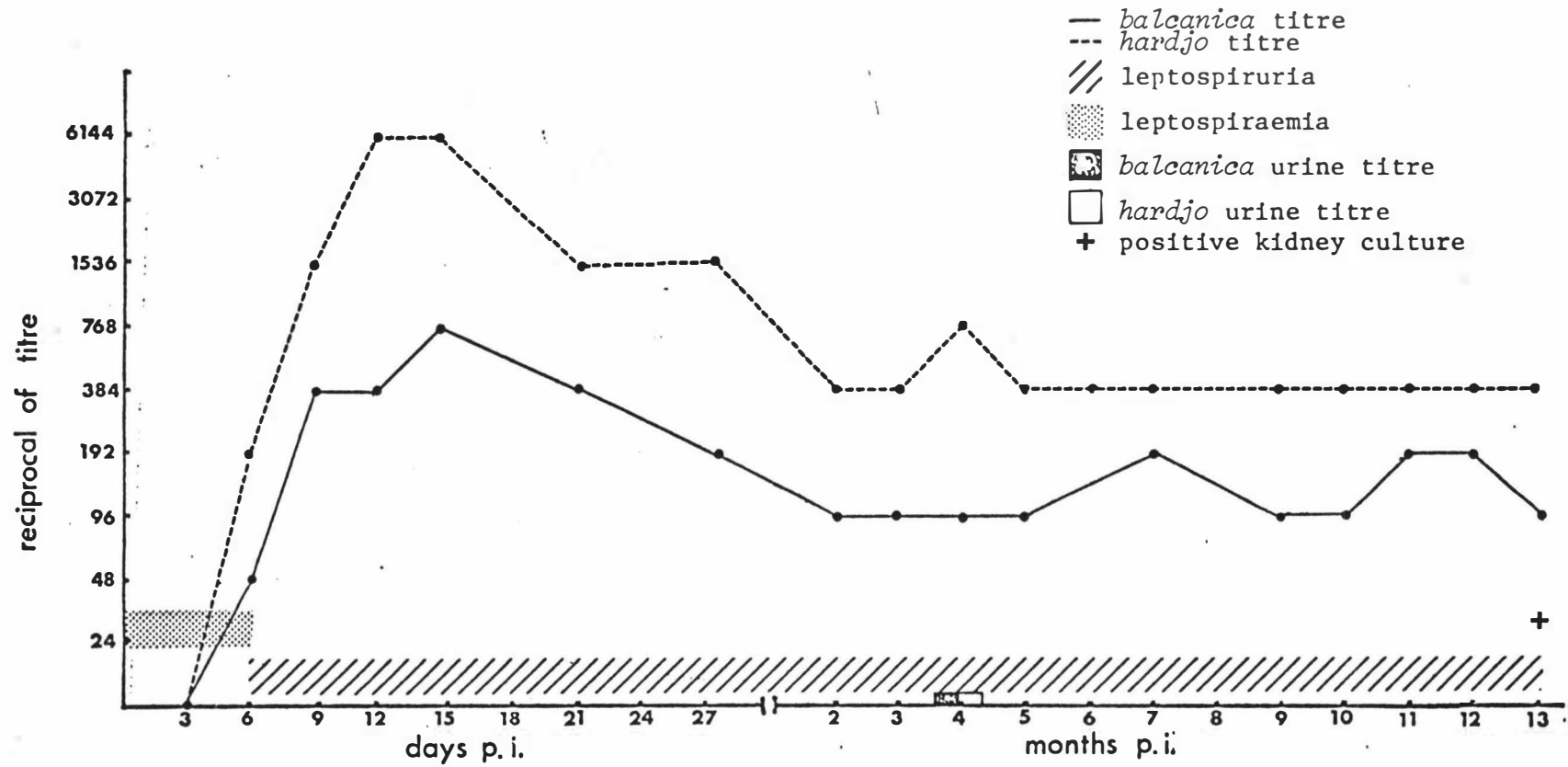
Figure 7.4. : Experimental infection with *balcanica* in possum E4.5.
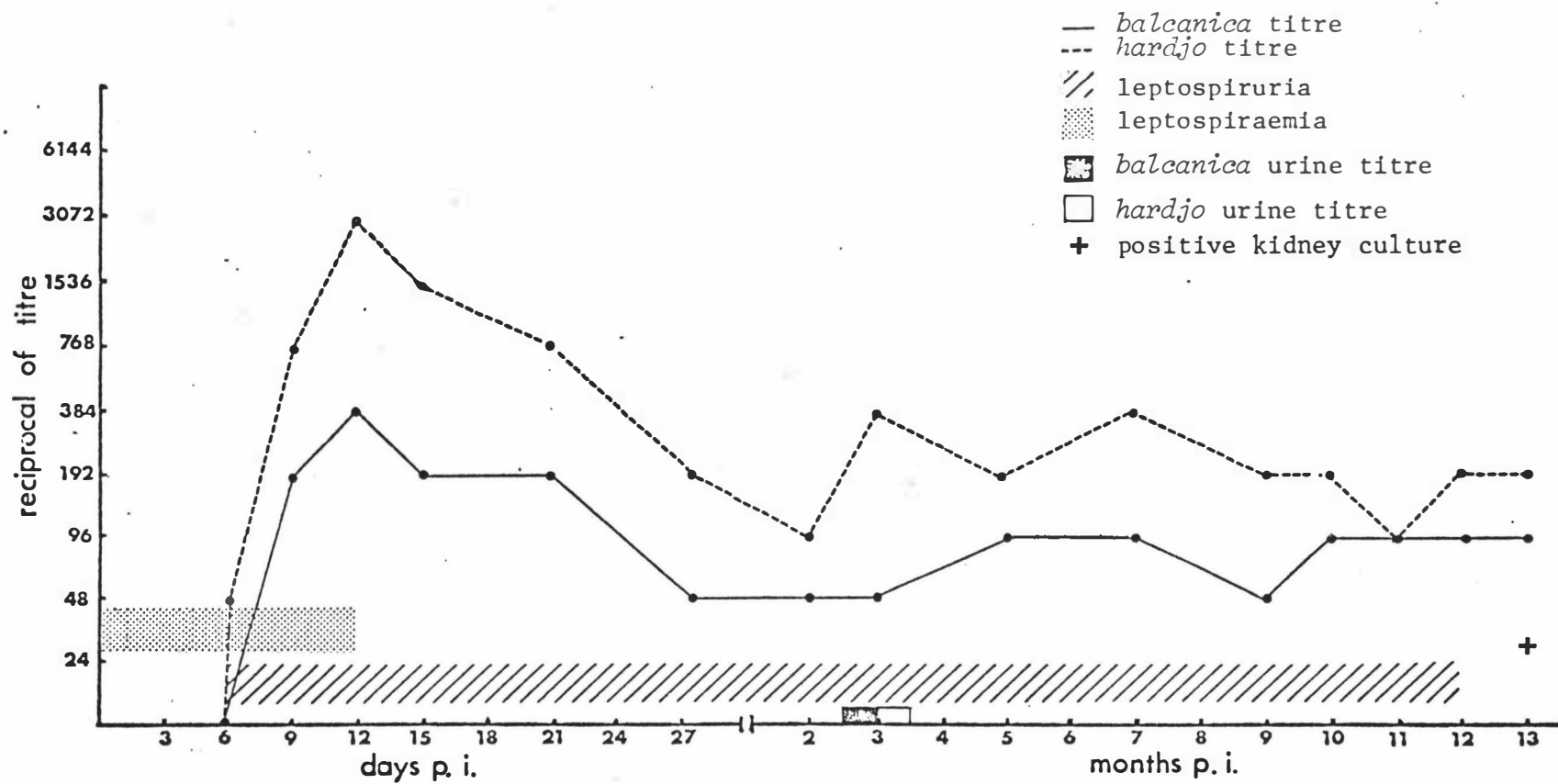
Figure 7.5. : Experimental infection with *balcanica* in possum E4.6.
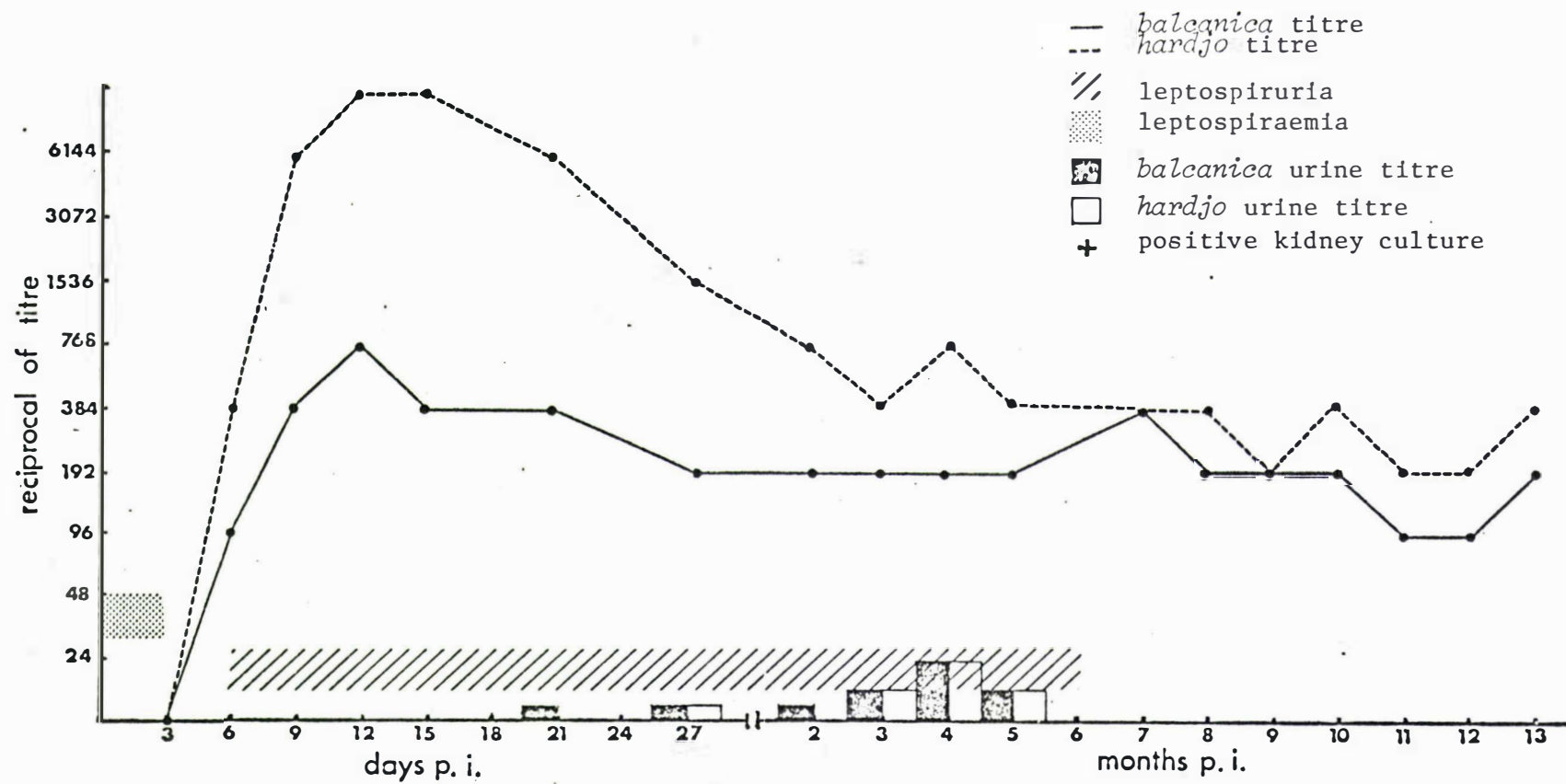
Figure 7.7. : Experimental infection with *balcanica* in possum E4.8.
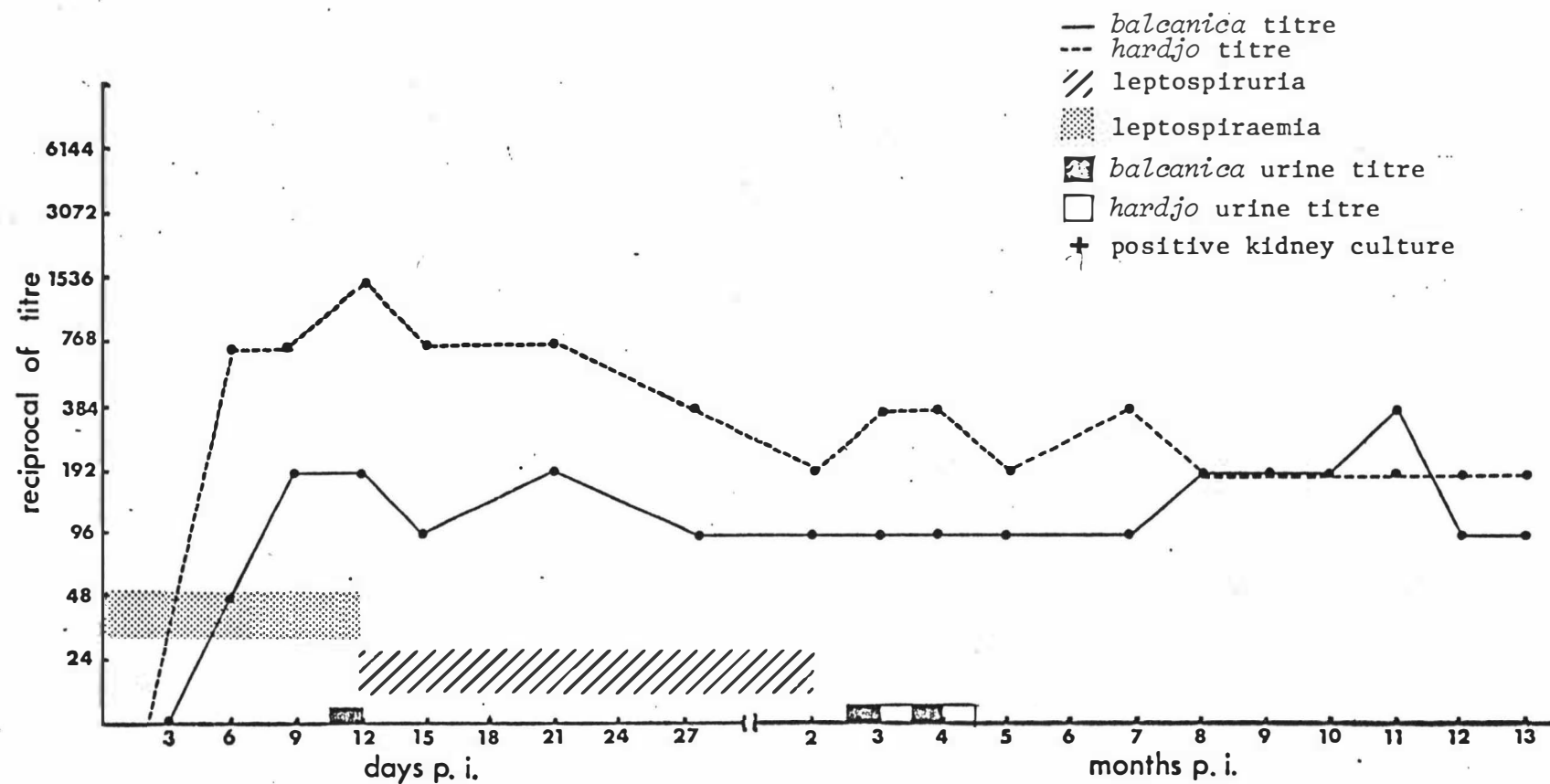
excreted for a period of 180 days following inoculation. It is interesting to note that leptospires were detected only once by dark-field microscopy in urine from possum E4.7.

Darkfield microscopy consistently revealed a change in the motility of leptospires in urine as the duration of infection increased. In general, the organisms were highly motile during the initial stage of leptospiruria but this motility was lost by day 60 p.i. The exception was urine from possum E4.5, in which low numbers of motile organisms were seen on day 270 p.i. The transitional stage between the shedding of motile and non-motile organisms often included an intermediate period in which the motility of leptospires was severely modified and consisted of very slow or aberrant movements.

There was considerable variation in the duration of leptospiruria in individual animals (Figures 7.2 to 7.7). Two of six possums were still shown to be shedding leptospires at the termination of the experiment at day 390 p.i. and kidney cultures were positive from these animals. Leptospires were not demonstrated in the urine of possum E4.5 at day 390 p.i. However, the kidney culture from this animal was positive at this time. Leptospiruria was not detected after day 180 p.i. in possum E4.6 and day 60 p.i. in possums E4.7 and E4.8. These latter three animals were culture negative at the termination of the experiment.

Urine titres to *hardjo* and *balcanica*, at a minimum urine dilution of 1:6, were low and sporadic, but were present in all possums between days 90 and 120 p.i. (Figures 7.2 to 7.7). The most consistent urinary titres occurred in possum E4.6 (Figure 7.4) and these peaked at day 120 p.i. Leptospiruria in this animal terminated shortly after the period in which agglutinins were detected in urine. Unlike serum titres, urine titres to *hardjo* and *balcanica* were equivalent in all except one sample. No evidence of leptospiral infection was detected in control animals.

A comparison of the different techniques used to detect leptospiraemia and leptospiruria in experimentally-infected possums is presented in Tables 7.4 and 7.5. During the lepto-

Table 7.3 : GMT's and log GMT's of Hebdomadis serogroup titres in possums experimentally-infected with *balcanica* in Experiment 4.

| Serovar | | 0 | 3 | 6 | 9 | 12 | 15 | 21 | 28 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 270 | 300 | 330 | 360 | 390 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | days post-inoculation | | | | | | | | | | | | | |
| *hardjo* | reciprocal of GMT | 0 | 0 | 239 | 2184 | 3461 | 2184 | 1378 | 675 | 388 | 436 | 446 | 308 | NT | 388 | 323 | 269 | 308 | 239 | 218 | 239 |
| | log GMT | 0 | 0 | 2.38 | 3.34 | 3.54 | 3.34 | 3.14 | 2.83 | 2.59 | 2.64 | 2.65 | 2.49 | NT | 2.59 | 2.51 | 2.43 | 2.49 | 2.38 | 2.34 | 2.38 |
| *balcanica* | reciprocal of GMT | 0 | 0 | 42 | 239 | 338 | 239 | 239 | 107 | 120 | 95 | 126 | 107 | NT | 151 | 135 | 95 | 135 | 135 | 95 | 95 |
| | log GMT | 0 | 0 | 1.62 | 2.38 | 2.53 | 2.38 | 2.38 | 2.03 | 2.08 | 1.98 | 2.10 | 2.03 | NT | 2.18 | 2.13 | 1.98 | 2.13 | 2.13 | 1.98 | 1.98 |

163

Figure 7.8. : Log GMT of *hardjo* and *balcanica* titres in experimentally-infected possums (Experiment 4).
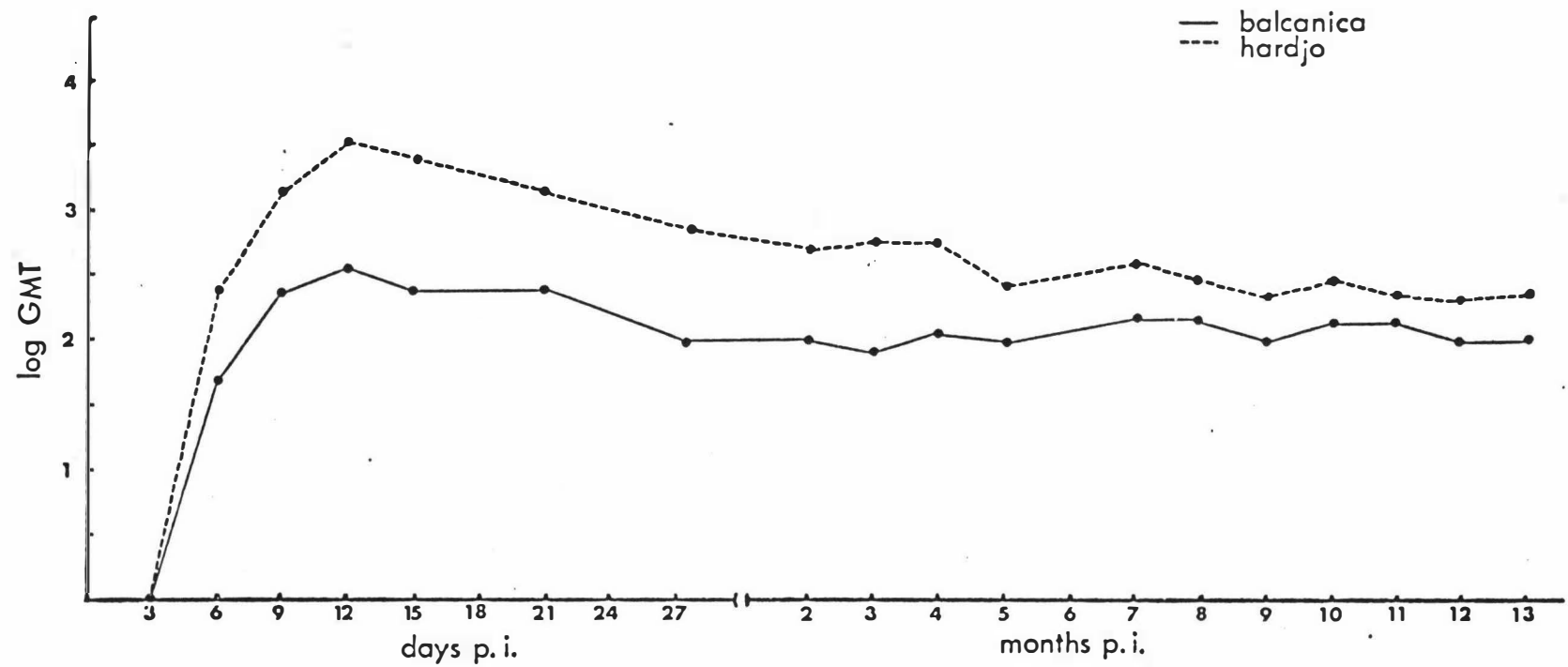
spiraemic phase of infection, direct culture in media and hamster incoulation were equally efficient methods for the isolation of leptospires from blood before the appearance of leptospiral agglutinins (Table 7.4). After the appearance of agglutinins (first recorded on day 6 p.i.) leptospires were isolated from direct blood cultures on only one occasion. In comparison, leptospires were recovered from hamsters inoculated with blood after this time on five occasions and a serological reaction was recorded in a sixth hamster.

Dark-field microscopy was the least sensitive of the three methods used to detect leptospiruria (Table 7.5) and demonstrated leptospires only during the peak shedding phase. This method failed to demonstrate leptospiruria on any occasion on which culture or hamster inoculation gave negative results.

Contamination of cultures limited the isolation of leptospires from urine inoculated into media containing 200 μg 5FU/ml. Despite this problem, isolates were obtained from three urine samples cultured in this medium (5% of culture-positive urine samples) that were not obtained from the same urine samples cultured in media containing 400 μg 5FU/ml. Of a total of 59 culture-positive urine samples, 56 isolations (95%) were in the media series containing 400 μg 5FU/ml and 27 (46%) were made in the media series containing 200 μg 5FU/ml.

Hamster inoculation of urine samples over the first 180 days of the experiment was slightly more efficient for the detection of leptospiruria than culture in media containing 400 μg 5FU/ml. Forty-six isolations were made from hamsters over this period whereas 43 isolations were made by direct culture. In two animals (E4.5 and E4.8), hamster inoculation detected an earlier onset of leptospiruria than direct culture. During the later stages of leptospiruruia, direct culture was more efficient than hamster inoculation for the isolation of leptospires.

Both direct urine culture in media containing 400 μg5FU/ml and urine inoculation of hamsters, the two most efficient methods for detecting leptospiruria, demonstrated a degree of intermittency in the isolation of leptospires from serial urine samples (Table 7.5).

Table 7.4 : Detection of leptospiraemia in experimentally-infected possums by culture and hamster inoculation in Experiment 4.

| Possum No. | Method | Day post-inoculation | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 3 | 6 | 9 | 12 | 15 | 21 | 28 |
| E4.1 | Culture | − | + | −D | | | | | |
| | Hamster inoc. | NT | + | + | | | | | |
| E4.2 | Culture | − | + | + | − | − | − | − | − |
| | Hamster inoc. | NT | + | + | − | − | − | − | NT |
| E4.3 | Culture | − | − | − | − | − | −D | | |
| | Hamster inoc. | NT | − | − | − | − | | | |
| E4.4 | Culture | − | + | − | − | − | − | − | − |
| | Hamster inoc. | NT | + | + | − | − | − | − | NT |
| E4.5 | Culture | − | + | − | − | − | − | − | − |
| | Hamster inoc. | NT | + | − | − | + | − | − | NT |
| E4.6 | Culture | − | + | − | − | − | − | − | − |
| | Hamster inoc | NT | + | − | − | − | − | − | NT |
| E4.7 | Culture | − | + | − | − | − | − | − | − |
| | Hamster inoc. | NT | + | − | − | − | +* | − | NT |
| E4.8 | Culture | − | + | − | − | − | − | − | − |
| | Hamster inoc. | NT | + | − | − | + | + | − | NT |

D = possum death

* = hamster serological response only.

Table 7.5 : Detection of leptospiruria in experimentally-infected possums by darkfield microscopy, culture and hamster inoculation (Experiment 4)

| Possum No. | Method used | 0 | 3 | 6 | 9 | 12 | 15 | 21 | 28 | 60 | 90 | 120 | 150 | 180 | 210 | 240 | 270 | 300 | 330 | 360 | 390 | Kidney culture |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| E 4.2 | DF microscopy | − | − | − | − | − | − | + | + | + | + | + | + | − | + | + | + | − | − | − | − | |
| | Culture 1 | − | + | + | − | − | + | − | + | − | + | + | − | − | + | − | − | − | + | + | − | + |
| | Culture 2 | − | − | + | − | + | + | + | + | + | + | − | − | − | + | − | + | + | + | + | + | |
| | Hamster inoc. | NT | − | + | + | + | + | + | + | + | + | + | + | − | − | − | NT | NT | NT | NT | NT | |
| E 4.4 | DF microscopy | − | − | − | − | − | − | + | + | + | + | + | − | + | − | − | − | − | − | − | − | |
| | Culture 1 | − | − | + | + | + | + | − | − | + | − | − | − | − | − | − | − | − | − | + | − | |
| | Culture 2 | − | − | + | + | − | + | + | + | + | + | + | + | + | − | − | + | − | + | + | + | + |
| | Hamster inoc. | NT | − | + | NT | + | + | + | + | + | + | + | − | − | − | − | NT | NT | NT | NT | NT | |
| E 4.5 | DF microscopy | − | − | − | − | − | − | NT | − | + | + | − | − | + | NT | − | + | − | − | − | − | |
| | Culture 1 | − | − | − | − | − | + | NT | + | − | + | − | + | − | NT | − | − | − | + | − | − | + |
| | Culture 2 | − | − | − | − | − | + | NT | + | + | + | + | + | + | NT | + | + | + | + | + | − | |
| | Hamster inoc. | NT | − | + | − | + | + | NT | + | + | + | + | − | − | NT | − | NT | NT | NT | NT | NT | |
| E 4.6 | DF microscopy | − | − | − | − | − | + | + | + | + | − | + | − | − | − | − | − | − | − | − | − | |
| | Culture 1 | − | − | − | + | + | − | − | + | − | − | − | − | + | − | − | − | − | − | − | − | − |
| | Culture 2 | − | − | + | + | + | + | + | + | − | + | + | − | + | + | − | − | − | − | − | − | |
| | Hamster inoc. | NT | − | + | + | + | + | − | + | + | + | + | + | + | − | − | NT | NT | NT | NT | NT | |
| E 4.7 | DF microscopy | − | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | − | − | − | − | |
| | Culture 1 | − | − | − | − | − | − | + | − | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | Culture 2 | − | − | − | − | − | − | + | + | + | + | − | − | − | − | − | − | − | − | − | − | |
| | Hamster inoc. | NT | − | − | − | + | + | + | + | + | + | − | − | − | − | − | NT | NT | NT | NT | NT | |
| 4.8 | DF microscopy | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | − | |
| | Culture 1 | − | − | − | − | − | − | + | + | − | − | − | − | − | − | − | − | − | − | − | − | − |
| | Culture 2 | − | − | − | − | − | − | + | + | + | − | − | − | − | − | − | − | − | − | − | − | |
| | Hamster inoc. | NT | − | − | − | + | + | NT | − | + | − | − | − | − | − | − | NT | NT | NT | NT | NT | |

culture 1 = EMJH + 200 µg 5FU/ml
culture 2 = EMJH + 400 µg 5FU/ml

Serial culture of urine from all animals between the times at
which leptospiruria was first and last detected by direct culture
produced isolates from 56 of 67 urine samples (84%).  Serial
inoculation of hamsters with urine from all possums, between
the times at which leptospiruria was first and last detected by
hamster inoculation, produced isolates from 43 of 46 pairs of
hamsters inoculated (93%).  Isolates were obtained by a combination
of both methods from all urine samples collected between the onset
and cessation of leptospiruria in all animals.

The results of urine inoculations in individual hamsters during
the first 180 days of Experiment 4 are given in Table 7.6 and
Figure 7.9.  The infectivity of urine inocula was maximal from day 15
to day 60 p.i. with more than 80% of each group of hamsters chall-
enged during this time becoming infected.  Urine inocula were the
most virulent for hamsters on days 15 and 21 p.i., with the highest
death rates and shortest mean death times occurring in the groups
of hamsters inoculated at these times. During the later stages of
leptospiruria in possums (day 90 p.i. on), the infectivity and
virulence of urine inocula for hamsters showed a marked decrease.

Histological examination of kidney sections stained by a
modified Warthin-Starry silver-impregnation technique failed to
detect leptospires.  In the kidney from possum E4.4 a few dark
granules, closely applied to the luminal surface of the tubular
epithelium, were seen.  This material may have represented
degenerated clumps of leptospires.

Haemotoxylin and eosin-stained sections of kidneys revealed
only very mild histological changes.  The kidney of possum E4.2
showed a very mild focal chronic interstitial nephritis with some
local proliferation of fibroblasts.  Occasional cellular casts
were present in renal tubules.  Sections from E4.4 showed a similar,
very mild interstitial nephritis.  Infiltration of small numbers
of lymphocytes, macrophages and plasma cells occurred predominantly
in the region of the loops of Henle and the collecting ducts.  There
was also some swelling of the cellular epithelium of nephrons in
this kidney, indicating that some nephrosis occurred in the distal
portions of the nephrons.  An occasional degenerate glomerulus was

Table 7.6 : Results of hamster inoculation with possum urine in Experiment 4.

| Possum No. | Hamster No. | Days post-inoculation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 9 | 12 | 15 | 21 | 28 | 60 | 90 | 120 | 150 | 180 |
| E4.2 | 1 | - | +(16)a | +(19) | +(17) | +(16) | +(14) | +(14) | +(15) | +(NF) | +(NF) | +(NF) | - |
| | 2 | - | +(17) | +(18) | +(18) | +(15) | +(14) | +(16) | +(NF) | +(17) | +(NF) | +(20) | - |
| E4.4 | 1 | - | +(13) | NT | +(NF) | +(13) | +(18) | +(17) | +(15) | - | +(NF) | - | +(19) |
| | 2 | - | +(NF) | NT | - | +(13) | +(16) | +(18) | +(16) | +(19) | +(NF) | - | +(NF) |
| E4.5 | 1 | - | - | - | - | +(NF) | NT | +(15) | +(NF) | +(17) | +(NF) | - | - |
| | 2 | - | +(NF) | - | +(NF) | +(NF) | NT | +(NF) | +(NF) | +(NF) | - | - | - |
| E4.6 | 1 | - | +(NF) | +(13) | +(14) | +(14) | - | +(NF) | +(17) | +(NF) | +(NF) | - | +(NF) |
| | 2 | - | +(NF) | +(NF) | +(NF) | +(14) | NT | +(NF) | +(15) | - | +(NF) | +(NF) | +(NF) |
| E4.7 | 1 | - | - | - | +(NF) | +(13) | +(13) | +(NF) | - | - | - | - | - |
| | 2 | - | - | - | - | +(15) | +(14) | +(NF) | +(NF)* | +(NF) | - | - | - |
| E4.8 | 1 | - | - | - | +(NF) | +(NF) | NT | - | +(NF) | - | - | - | - |
| | 2 | - | - | - | - | - | NT | - | - | - | - | - | - |

+ = culture positive    a = time until death    NF = non fatal infection    * serological response only.

Figure 7.9. : Parameters of infection with *balcanica* in hamsters inoculated with serial samples of possum urine in Experiment 4.
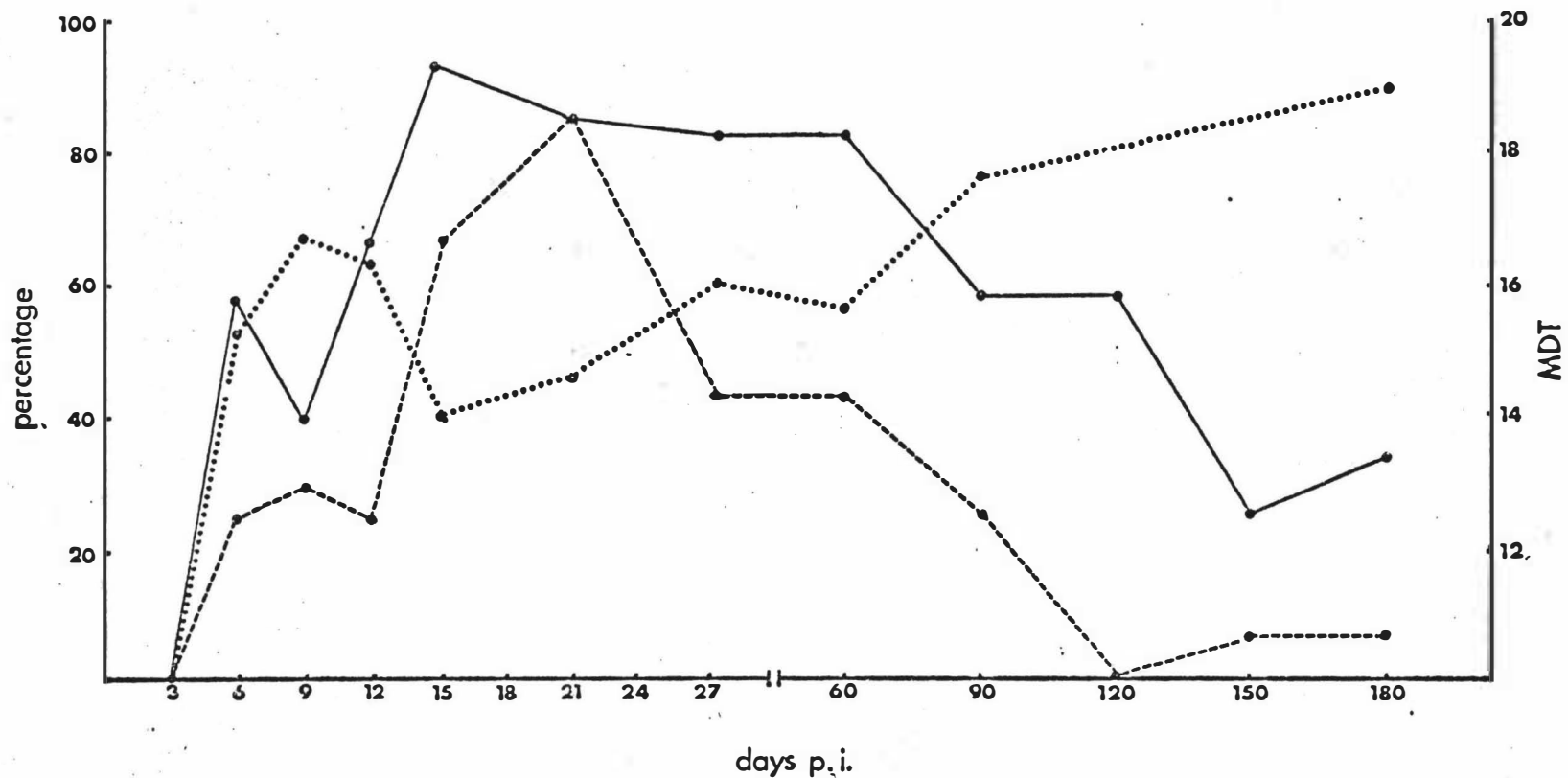
—— percentage of inoculated hamsters culturally or serologically positive
---- % of inoculated hamsters that died
••••• mean death time (MDT)

seen.  No significant inflammatory changes were seen in kidney
sections from the remaining possums.  Some degenerated epithelial
cells and protenaceous casts were present in a small number of
nephrons in possum E4.6.  A tinctorial change was noted in the
cellular epithelium of some tubules in possum E4.7 and there was
a transition from cuboidal to columnar epithelium in the papillae
and pelvis of this kidney.

The *balcanica* culture used for inoculation of possums in
Experiment 4 was shown to have a similar infectivity and virulence
for hamsters as the cultures used in the previous infectivity
experiments.  All six hamsters inoculated died by day 14 p.i. and
the mean death time was 13.2 days.

Inoculation of possums with serovar *hardjo* (Experiment 5)
produced no serological or cultural evidence of infection.  The *hardjo*
cultures used had varying infectivity for hamsters.  Hamsters appeared
to be insusceptible to infection with strain NHI 2/309, a human isolate
that had been maintained in liquid media for two years.  Although low
titres to *hardjo* were recorded in 3 of 8 hamsters inoculated with this
serovar, no isolations were made from kidneys at day 21 p.i.  All of
the hamsters inoculated with isolates obtained from bovine urine
(strains 1 and 2) developed *hardjo* titres and isolations were made from
six hamsters in this group (75%).  No clinical symptoms were observed
in any of the infected animals.  The leptospires present in the bovine
urine sample failed to infect hamsters.

The serological response of possums to inoculation with Ballum
serogroup isolates from a rat and a mouse is shown in Table 7.7.
All four possums challenged developed *ballum* titres.  The maximum
titre recorded was 1:384 in possum E6.1.  This possum was the only
animal which was culture-positive at day 21. p.i.  Dark-field
microscopy and urine culture failed to detect leptospiruria in any
animals.

The Ballum serogroup isolates were highly infective and virulent
for hamsters, with all inoculated hamsters dying on days 4 or 5 p.i.

Table 7.7 : Serological response of possums to inoculation with
leptospires of the Ballum serogroup.

| Possum No. | *Ballum* titre | | | Culture |
|---|---|---|---|---|
| | 7 days p.i. | 14 days p.i. | 21 days p.i. | (21 days p.i) |
| E6.1 | 1:96 | 1:384 | 1:192 | + |
| E6.2 | 0 | 1:96 | 1:96 | − |
| E6.3 | 1:96 | 1:192 | NT | − |
| E6.4 | 1:24 | 1:96 | 1:48 | − |

## DISCUSSION

The pathogenesis of *balcanica* infection in experimentally-infected possums is characteristic of leptospiral infection in a maintenance host. Possums were infected with moderate numbers of organisms by the IP route and high peak titres in the early stage of infection were followed by a sustained serological response. Infected animals showed no clinical symptoms and underwent a greater weight gain during the course of the experiment than would be expected in the field. Hamster inoculation demonstrated that infective organisms were shed in urine for at least 180 days and serial culture of urine demonstrated that leptospiruria persisted for at least a year in 50% of the animals monitored. Despite the fact that up to $10^6$ organisms/ml of urine were shed by infected animals, histological changes in kidneys were very mild. Thus the experimental *balcanica* infections confirmed the definition made initially from field serological and cultural data that the possum is a maintenance host for *balcanica*.

The serological response of infected animals was similar to that reported by other workers investigating leptospirosis in maintenance host species for a particular serovar (Morse *et al*,1958; Fennestad, 1963; Taylor *et al*,1970; Ellis and Michna,1977 ). Titres of individual animals, both within and between experimental groups, showed some degree of variation in peak titres and levels at which titres persisted. Such variation is common in experimental infections (Morse *et al*,1958; Fennestad, 1963; McGowan and Karstad, 1965; Reilly, 1970) and individual variation in titres has also been shown to occur in natural outbreaks of leptospirosis (Hoare and Claxton, 1972; Hodges, 1977; Hellstrom, 1978). The within-group variation in titres in these experimental studies is probably due to a variation in the immune response of individuals, whereas the between-group variation may reflect differences in infectivity and virulence of the infecting organisms. Hocker and Bauer (1965) found that peak titres in experimentally-infected rabbits were proportional to the dosage of leptospires and each ten-fold increase in the inoculum resulted in a two-fold increase in titres; however the relationship between serological response and infecting dose is far from constant. Minette and Shaffer (1968) reported that

titres in experimentally-infected monkeys were not proportional to numbers of infecting organisms and similar results were reported in several experimentally-infected, free-living species by Reilly (1970).

The persistence of titres for long periods in possums in Experiment 4 provides an explanation for the high prevalence of titres found in cross-sectional surveys of mature possums in free-living populations. Titres monitored after day 60 p.i. showed very little change compared with the rapid logarithmic decay of peak titres that occurred before this time. This constant nature of long-term titres prevented the calculation of a regression co-efficient for the decrease of titres with time of infection.

A transient serological response to infection was detected in possum E2.5. Agglutinins were present for only a very short time in this animal, yet leptospires were recovered by kidney culture at day 70 p.i.

Culture-positive but sero-negative animals occur in many populations with endemic leptospiral infection, even when titres are read at low serum dilutions (Alexander *et al* , 1963; Martin *et al*, 1967; Twigg *et al*, 1968; Hirschberg and Vaughn, 1973). It is possible that a transient serological response in some animals, as observed in possum E2.5, may contribute to the number of animals in this category. The occurrence of sero-negative carriers also infers that leptospires localised in renal tubules exert little, if any, antigenic stimulus on the host.

There was no correlation at the termination of experiments E2 and E4 between the level of titres of individual animals and presence of leptospires in the kidneys. The duration of leptospiruria was similarly unrelated to the level of titres. Similar findings were reported by Ellis and Michna (1977) in experimental Hebdomadis serogroup infection in heifers. Thus the level of humoral antibody

does not appear to be correlated with the cessation of leptospiruria
and elimination of leptospires from the kidney.

Little is known about the occurrence of clinical symptoms in
leptospiral infection in wildlife (Roth, 1970). The lack of
symptoms in possums and the failure of clinical pathological
aids to demonstrate disruption of normal renal physiology showed
that although *balcanica* was highly infective for possums, the
pathogenicity was low. The severe haemoglobinuric nephrosis that
occurred in hamsters infected with *balcanica* did not occur in possums.
Differences in the pathogenicity of a particular serovar for
different animal species are well recognised (van der Hoeden,1954;
Reilly, 1970; Chernukcha *et al*,1974; Tripathy and Hanson,1974).
Histological examination of possum kidneys did not reveal the
large aggregations of inflammatory cells that often characterise
chronic leptospiral intersitial nephritis in other species (Tabel,
1970; Taylor *et al*,1970; Ryan, 1978).

Leptospiraemia was shown to be of up to 15 days duration.
The termination of leptospiraemia is generally regarded to be due
to the appearance of leptospiral antibodies in the blood and most
investigators have reported this to occur 6 to 8 days p.i. (Taylor,
*et al*,1970; Hanson *et al*,1971; Ellis and Michna, 1977 ). It has
been shown in some cases, however, that leptospires can survive in
the presence of high levels of circulating antibodies. Taylor
*et al* (1970) reported that two of ten dogs experimentally infected
with *canicola* had a recurrent leptospiraemia at days 25 and 28 p.i.
respectively and Schricker and Hanson (1961), who treated infected
guinea pigs with large doses of cortisone, found that a recurrent
leptospiraemia could occur in spite of high levels of circulating
antibody. Hamster inoculation of blood samples in the present
study showed that leptospires were present in the blood in 50%
of possums on days 12 and 15 p.i., by which time peak antibody
titres had been reached. Leptospires were therefore capable of
survival in the presence of high levels of humoral antibody,
indicating that the termination of leptospiraemia has a more
complex basis than the mere appearance of immunoglobulins that
are measured by the MAT. It was also demonstrated that hamster
inoculation was a much more sensitive method than direct culture
for detecting the true duration of leptospiraemia. Direct culture

failed to detect leptospiraemia after day 3 p.i. in all but one
sample. This may have been due to the larger inocula received
by hamsters or alternatively some growth-inhibiting factor may
have been transferred in blood inoculated directly into media.
Previous workers, relying on direct culture, may have under-
estimated the true duration of leptospiraemia and therefore
incorrectly attributed the apparent termination as being due to
the appearance of circulating antibody.

Leptospiruria in possums infected with the same inocula in
Experiment 4 was very variable, however 50% of the animals were
still excreting organisms one year after infection. The duration
of excretion of particular serovars by their recognised maintenance
hosts is often characterised by such variability (Morse *et al*, 1958;
Chernukcha *et al*,1974; Hanson, 1976) and long term excretion is not
only restricted to animals that are recognised as maintenance hosts
for a particular serovar (Roth *et al*,1963; Michna, 1970). The cause
of these variations in the duration of leptospiruria is unknown.
Leptospiruria of more than a year's duration has been documented
in several free-living species (Babudieri, 1958; Roth *et al*,1963;
van der Hoeden, 1964; Tripathy and Hanson,1976) and thus the
continued transmission of leptospiral infection within an endemic
focus is ensured. Leptospururia in domestic animals has generally
been shown to be of less than a year's duration (Morse *et al*,1958;
Sullivan, 1970; Hanson,1976; Hellstrom, 1978).

Darkfield microscopy, although a relatively insensitive method
for the detection of leptospiruria, provided valuable information
on the intensity of leptospururia and the motility of shed organisms.
Leptospires in urine lost their motility soon after the period
of most intense leptospururia, however urine inocula containing
non-motile organisms were still infective for hamsters and
isolates were also obtained by direct culture of these samples.
Loss of motility was therefore not associated with a loss of
infectivity or viability.

The demonstrated insensitivity of dark-field microscopy for the
detection of leptospiruria questions the conclusions of workers who
have used this technique alone to determine the time of onset,

constancy and duration of leptospiruria in different species
(Webster,1955; Sullivan,1970; Hanson,1971; Ellis and Michna,1977 ).
The present study demonstrated that hamster inoculation and direct
culture of urine samples detected leptospiruria in the majority of
animals on day six p.i., whereas the majority of positive ident-
ifications by darkfield microscopy were not made until day 21 p.i.
(The above-mentioned authors reported that leptospiruria in a
variety of species, as detected by dark-field microscopy, began
on post-inoculation days 13 to 16, 24 to 30, 12 to 40 and 22
respectively.)

Although intermittent shedding of leptospires has been widely
reported to be a characteristic of leptospiruria in a variety of
species, investigations have never provided an explanation for
this characteristic. It is possible that these reports of inter-
mittent shedding have reflected the insensitivity of monitoring
techniques. When isolation results from direct culture in media
containing 400 µg 5FU/ml and hamster inoculation were combined
in the present study it was evident that leptospiruria was constant
in all animals,yet if only one technique was considered,intermittent
results were obtained. Hamsters were the most sensitive means of
monitoring leptospiruria over the first 180 days of infection,
however they have rarely been used for this purpose by other
workers. As discussed later, this study also demonstrated that both
dead and surviving hamsters must be cultured to ensure accurate
results.

The presence of leptospiral antibody in the urine of infected
animals has been reported by many workers (Stuart,1956; Morse *et al*,
1958; Faine, 1962; Doherty,1966; Hirschberg and Vaughn,1973). Titres
are generally low,and variable between different species. Stuart
(1956) found that urine titres in humans with Weil's disease were,
on average, 1% of serum titres. Faine (1962) found that urine
titres in mice infected with *australis* were up to 17% of serum titres
and Hirschberg and Vaughn (1973) were able to demonstrate leptospiral
antibodies only in concentrated bovine urine. Titres in the present
study ranged from 1:6 to 1:24 and were similar for both *hardjo* and
*balcanica*. These low titres were, on average, 0.5% of serum *hardjo*
titres and 5% of serum *balcanica* titres.

It is probable that urinary antibody is locally-produced in the kidney. Five of eighteen kidney homogenates tested in the present study (28%) had similar titres to the serum titres of the same animal and several had lesser titres. These homogenate titres were unlikely to be due to residual blood in kidney tissue, as residual blood left after the animal had been bled out would be a very small proportion of the total kidney mass. In addition the kidney mass was diluted 20% w/v with SBM during the homogenation process. The minimal contribution of residual blood to homogenate titres was confirmed by the passive intravenous immunisation of two possums with high titre antiserum. The ratios of serum to homogenate titres in these animals, 24 hours after immunisation, were 20:1 and 12:1 respectively. This indicated that residual blood in kidneys contributed only 5 to 8% of homogenate titres. Control animals were provided by pouched juveniles with passively-derived, maternal antibody titres. The kidney homogenates from these animals were negative, thus indicating that infection is necessary for the local production of antibody in kidneys.

Morse et al(1958) and Tabel (1970) were also of the opinion that leptospiral antibodies in the urine were produced in the kidney. They found that antibodies in the urine of pigs and skunks infected with pomona peaked at 3 - 4 months p.i., by which time serum titres had markedly decreased from the peak levels reached soon after infection. This indicated that urine titres were independant of serum titres. A similar pattern was observed in possums in the present investigation. Tabel (1970) also noted that there were large numbers of plasma cells in the kidneys of chronically infected skunks and he considered that these cells may have been responsible for local antibody production. Due to the very mild inflammatory changes in kidneys of chronically-infected possums however,plasma cells were present in only small numbers.

The influence of urinary antibody on leptospires shed in urine is open to question. Stuart (1956) considered that urinary antibody had a deletarious effect as, when heat-killed leptospires were added to urine, there was an increase in the number of recoveries. Morse et al(1958) and Killinger et al(1976) were of the same opinion and Hanson (1977) suggested that leptospires in the urine

of chronically infected bovines were less virulent than those
in urine during the acute stage of infection, due to the presence
of antibodies.  Contrary opinions have been expressed by other
workers.  Faine (1962) considered that renal leptospires were
resistant to leptospiral antibody and Doherty (1966) failed to find
a significant difference in guinea pig infectivity when leptospiruric
bovine urine samples, with and without *pomona* antibodies, were used
as inocula.

The present study did little to alleviate this controversy.  It
was observed by dark-field microscopy that aggregations of particulate
matter were sometimes present on leptospires in urine after day
60 p.i.  This may have been due to the agglutinating effect of
adherent leptospiral agglutinins, however these organisms remained
infective for hamsters.  The presence of urinary antibody appeared
to have no correlation with the duration of leptospiruria, although
in possum E4.3 a higher than average urinary antibody peak was
followed by termination of leptospiruria.  Hamsters infected with
urine from long-term shedders experienced a longer mean death time
and a lower death rate than hamsters infected with urine in the
initial stages of leptospiruria.  This may have been due to an
antibody-related decrease in virulence of leptospires in the urine
of chronically-infected possums, however, it is more like that
these findings were due to the lower number of leptospires in urine
inocula.  It has been shown by other workers that the mean death
time of infected hamsters is inversely related to the number of
infecting organisms (Baker and Baker, 1970; Ellinghausen and
Painter, 1976).  These workers also demonstrated that when the
number of infecting organisms is low, there is a decrease in the
number of deaths in a group of infected hamsters.  The number  of
leptospires in urine inocula was not the only factor affecting
death rate and mean death time in the present study as the time
of the highest death rate and shortest mean death time of infected
hamsters preceeded the period when leptospiruria was of maximum
intensity.

Three of 17 possums challenged intraperitoneally with *balcanica*
in the present study were refractory to infection.  A titre of 1:96

to *balcanica* was present in the pre-inoculation sera from possum
E2.3 and a titre of 1:24 was recorded in possum E3.4 in the first
pre-inoculation screen, 28 days before challenge, but not in the
second.  These titres probably represented maternally-derived
antibody and, although low, they appeared to confer protection
against infection.  Pre-inoculation titres were not present in
possum E4.3.  Similar results were reported in experimental
challenge of hiefers with an Hebdomadis serogroup organism by
Ellis and Michna (1977b).  These workers were unable to infect
8 of 20 animals in the experimental group, some of which had low
titres pre-inoculation.  The presence of a protection factor in
juvenile possums that is not measurable by the MAT is investigated
in Chapter VIII.

The *balcanica* cultures used as inocula in the four infectivity
experiments were of similar infectivity and virulence for hamsters
and therefore it was assumed that all experimental groups received
a comparable challenge.  The absence of any evidence of infection
in possums challenged by the percutaneous route suggested that
they were insusceptible to challenge by this route.  It was shown
in Chapter V that there was a ten-fold increase in the minimum
infective dose (MID) for hamsters infected by the percutaneous
compared with the intraperitoneal route and other workers have
also found that the route of infection affects the MID of leptospires
in a variety of species (Fennestad, 1963; Sullivan, 1970; Kiktenko
*et al*, 1976).  The inflammatory reaction which would have been
induced by abraiding the skin may have also affected challenge by
this route.

An alternative possibility is that these animals (approximately
7 - 8 months of age) may have still had a degree of maternally-
derived immunity, undetectable by the MAT.  This is discussed in
Chapter VIII.

Possums were insusceptible to experimental infection with sero-
var *hardjo*.  *Hardjo* inocula from varying sources were used and the
bovine urine isolates were infective for 75% of hamsters inoculated.
Serovar *hardjo* is generally considered to be non-pathogenic and of
low infectivity for hamsters and therefore the high hamster infect-
ion rate achieved with the bovine isolates indicated that possums

received a very significant challenge. It has been found that young animals of an accidental host species for a particular serovar are more susceptible to infection than old animals (Webster, 1955; Chernukcha *et al*,1974) and therefore the juvenile possums used in the present investigation would theoretically be the most susceptible if *hardjo* was infective for possums. The experimental results support the findings of the cultural survey and indicate that possums are refractory to challenge by serovar *hardjo*. This compliments the findings of overseas workers who have been unable to find *hardjo* infection in other free-living species that share a common environment with bovines that have endemic *hardjo* infection (Sullivan, 1974; Hanson,1976).

The marked difference in susceptibility of possums to *balcanica* compared with *hardjo*, two strains closely related antigenically, indicates that the biological properties of different leptospiral serovars are independent of antigenic properties.

Experimental infection in possums with organisms of the Ballum serogroup was characteristic of infection in an accidental host. An isolate was recovered from only one animal on day 21 p.i. and the absence of a detectable leptospiruria in any animals indicated that the possum was a dead-end host. The very low prevalence of Ballum serogroup titres found in the serological survey of field populations (Chapter IV) supports these experimental findings.

## SUMMARY AND CONCLUSIONS

1.  Experimental infection with *balcanica* in possums was character-
    istic of leptospiral infection in a maintenance host.

2.  Although there was some individual variation in the serological
    response to infection, titres persisted at moderate levels for
    390 days in Experiment 4.

3.  There was no correlation between the level of humoral antibody
    and the termination of leptospiraemia and leptospiruria in
    individual animals.

4.  Leptospiruria was of more than a year's duration in 50% of
    infected animals.

5.  While there was a loss of motility of leptospires in urine after
    a period of peak shedding, this was not associated with a loss
    of infectivity or viability for hamsters.

6.  Dark-field microscopy was an insensitive technique for the
    detection of leptospiruria.

7.  A combination of results from hamster inoculation and direct
    culture in media containing 400 µg 5FU/ml demonstrated that
    leptospiruria was constant in all animals throughout the
    period monitored.

8.  Low urinary antibody titres were found in all infected animals.
    These were independent of serum titres and it is probable that
    urinary antibody is locally produced in the kidney.  This
    hypothesis was supported by passive immunisation studies.

9.  Low levels of residual maternal antibodies protected juvenile
    animals from experimental challenge.

10.  Possums were insusceptible to infection with *hardjo*.  This
    complemented the results of the field cultural survey.

11. Experimental infection with leptospires of the Ballum serogroup was characteristic of infection in an accidental host.

CHAPTER VIII


SOME ASPECTS OF THE EPIDEMIOLOGY OF

*BALCANICA* INFECTION IN POSSUMS


## INTRODUCTION

It has been stated by Sveshnikova and Chernukha (1972) that the requirements for the development of a new nidus of leptospirosis are (i) a favourable host habitat, (ii) a favourable host biology for transfer of infection, and (iii) a large number of hosts inhabiting a wide territory. The introduction of the possum to New Zealand resulted in a rapid colonisation of vacant ecological niches and it has been shown that a nidus of *balcanica* infection has become established in pastoral possum populations (Chaptver VI). Two features of this nidus are the high prevalence of infection in pastoral populations from a variety of locations (Chapters IV and VI) and the marked difference in age-specific prevalences of infection.

The survival of endemic leptospiral infection in a maintenance host species depends on the continued transmission of the serovar, either directly, or indirectly via the environment. It has been reported that favourable environmental conditions for survival of leptospires facilitate the transmission of infection from free-living maintenance host populations to domestic stock and man (Alston and Broom, 1958; Babudieri, 1958; Alexander *et al*, 1963; Schnurrenberger *et al*, 1970; Twigg *et al*, 1972). Favourable environmental conditions for survival include slightly alkaline conditions, adequate moisture, moderate temperatures and lack of sunlight; whereas unfavourable conditions include markedly acid or alkaline conditions, lack of moisture, high or low temperatures, sunlight and high salt concentrations (Okazaki and Ringen, 1957; van der Hoeden, 1958; Gordon-Smith Turner, 1961; Baker and Baker, 1970; Karaseva *et al*, 1973; Hellstrom, 1978). A number of workers are of the opinion that

favourable enviromental conditions for the survival of leptospires
are also important in the maintenance of endemic infection with a
particular serovar within a maintenance host species (Ferris *et al*, 1961;
Gordon-Smith *et al*, 1961b; Andrews and Ferris, 1966; Turner, 1967;
Twigg *et al*, 1969). These workers have generally regarded rainfall
and water content of soil to be most important in this respect. Other
workers have been unable to show an association between wetness of the
environment and prevalence of infection with a particular serovar in
some maintenance host populations (Twigg *et al*, 1968; Schnurrenberger
*et al*, 1970).

It has also been postulated that population factors are of more
importance than environmental conditions favouring the survival of
leptospires in the maintenance of endemic infection in some species
(Blood *et al*, 1963: Trainer *et al*, 1963; Turner, 1967; quoting Wolff,
1963). In one of the few comparative studies of the same species
of animal inhabiting different ecosystems (Brown and Gorman, 1960),
it was found that the present of endemic *ballum* infection in house
mice from a wide range of habitats was almost exclusively restricted
to those habitats that supported significant numbers of mice.

The fact that the factors that enable an ecosystem to support
a natural concentration of a free-living species may also favour the
environmental survival of leptospires compounds the problem of
determining the relative importance of population density and a
favourable environment in the maintenance of a high prevalence of
infection in an endemic focus. It can be concluded from the literature
that it is largely undetermined whether transmission within a
maintenance host population is via the environment or by direct
contact between animals.

This chapter describes an attempt to elucidate some of the factors
affecting the prevalence of *balcanica* infection in different possum
populations. Sampling locations were chosen so as to represent as
diverse environmental conditions as possible, yet retain some
geographical proximity to pastoral locations where the prevalence of

infection was known. Estimates of possum population densities are difficult to establish and require many man-hours (see Chapter II) and therefore only subjective indices of relative abundance were used in this investigation.

An investigation of the marked age-specific difference in prevalence of infection that occurred in all pastoral possum populations sampled (Chapters IV and VI) is also described. It has been shown that infection does not become established in possums until the onset of sexual maturity. It is well established that maternally-derived antibodies protect neonates against leptospiral infection, however, possums do not reach sexual maturity until at least one year of age (Chapter II). The investigation of the lack of infection in juvenile animals involved a determination of the level and persistence of maternally-derived antibodies, the leptospiral growth inhibiting activity of sera from juveniles and studies in experimental animals.

MATERIALS AND METHODS

Description of sampling locations

The possum populations sampled inhabited three distinctly different ecosystems : coastal seaface, deep forest and forest-pasture boundary. The prevalence of *balcanica* infection in these populations was compared with that of pastoral possum populations. The general description and soil types of each location from which possums were sampled is given in Table 8.1. Geographical locations are shown in Figure 8.1.

1. Coastal seaface

Two samples were obtained from Pukerua Bay and Makara in July, 1977. Both locations face the prevailing south-west winds and are therefore exposed to high levels of wind-borne salt water. Soil samples were taken for analysis. Because of the very steep cliff face and the dessicating effect of onshore winds, the surface environment was very dry except during periods of rain.

Table 8.1 : Description of locations from which possum populations were sampled

| Location | Description | Vegetation | Rainfall (mm) | Soil type | Soil description | Soil pH |
|---|---|---|---|---|---|---|
| Pukerua Bay | Eroded coastal cliffs | Stunted scrub and grass | 1000-1500 | Makara stony loam | Skeletal, clay loam derived from Greywacke | 5.6 - 5.8 |
| Makara | " | " | " | " | " | " |
| Manganhao | Mountain range | Mixed native forest | 2500 | Ruahine steep-land soils | Skeletal, derived from Greywacke | 5.5 |
| Maunga-taniwha | High country river terrace | Broadleaf-podocarp forest | 2200-2500 | Urewera sandy silt | Rhyolitic ash on Greywacke | 5.5 - 6.0 |
| Akatarawa | Broken hill country | Cut-over forest | 1500 | Ruahine steep-land soils | Skeletal, derived from Greywacke | 5.6 |
| Pakaututu | Hill country | Forest-pasture boundary | 1500 | Kaweka sandy silt | Rhyolitic ash on Greywacke | 5.8 |
| Pauatahanui | " | " | 1500 | Ruahine steep-land soils | Skeletal, derived from Greywacke | 5.6 |
| Dannevirke | " | " | 1650 | Takapari hill soils and Dannevirke silt loam | Mixed | 5.5 |

Figure 8.1. : Geographical locations from which possums inhabiting deep-forest, forest-pasture boundary and coastal seaface ecosystems were sampled.

Despite the seemingly inhospitable environment, the relative abundance of possums was high. Possums were taken by cyanide poisoning.

2.   Deep forest

The location criteria for deep forest samples were (i) more than four miles from the nearest farmland, (ii) the absence of feral cattle and sheep and (iii) a forest area which supported possum populations of moderate or high density.

Two field trips into the catchment of the Mangahao River, in May and September 1977, resulted in only 14 possums being taken by cyanide poisoning. Heavy rain occurred on both occasions and the small numbers taken were a reflection on the weather conditions rather than low population density.

Possums from Maungataniwha were taken by cyanide poisoning and trapping on the northern and southern terraces of the Waiau River (southern boundary of the Urewera National Park) in October, 1977 and March, 1978. The relative abundance of possums on the northern side of the river (sample 1) was high. The relative abundance on the souther side was moderate, as commercial operators had been active in this area for several years.

The third deep forest sample was taken from the Akatarawa forest by trapping and poisoning in August, 1977 and February, 1978. The relative abundance of possums was moderate.

3.   Forest-pasture boundary

These sampling locations were chosen, wherever practicable, to be in the same geographical region as deep forest and pasture locations where the prevalence of *balcanica* infection was known. Trap and poison lines were set along the forest-pasture interface.

The Pauatahanui sample was taken by cyanide poisoning in July, 1978. The broken hill country in this area is grazed by sheep and cattle. The relative abundance of possums was moderate. Commercial

trappers operated intermittently in this area.

Pakaututu is located 40 miles south of Maungataniwha. Sheep
are grazed on steep hill country. Possums, present in moderate
numbers, were taken by trapping and poisoning in June, 1977.

The Dannevirke forest-pasture boundary sample was taken by
night-shooting along the eastern side of the Ruahine ranges in March
and April, 1977. Sheep and cattle were present on grazing land.
The relative abundance of possums was high.

Sera were tested against *balcanica* antigen, at a minimum serum
dilution of 1:24, as previously described. A random sample of 100
sera from possums inhabiting deep forest and coastal seaface
ecosystems were also tested against *ballum*, *copenhageni*, *pomona* and
*tarassovi* antigens.

## Investigation of maternally-derived antibodies in sera from juvenile possums.

Sera from 104 juveniles were tested against *balcanica* antigen
to determine the level and persistence of maternally-derived antibody
titres. The sample consisted of pouched-young, juveniles that had
left the pouch but were still living in association with their dams,
and independant juveniles. All animals were taken from the Woodville
and Dannevirke regions between March and October, 1976.

The juvenile possums were weighed and the age of those weighing
less than 900 g was determined from the nomogram constructed by Lyne
and Verhagen (1957) (Figure 8.2). Juveniles weighing more than 900 g
could not be aged by this nomogram.

Kidneys from all juvenile possums were cultured according to
standard techniques.

Plate 8.1. : Coastal seaface ecosystem.

Plate 8.2. : Deep forest ecosystem.

Plate 8.3. : Forest-pasture boundary ecosystem

Growth inhibiting activity of sera from juvenile animals

The leptospiral growth inhibition test used was a modification of that described by Tripathy *et al* (1973)(see Appendix IV). A random sample of sera from ten juveniles weighing less than 900 g and ten juveniles weighing more than 1200 g were tested for leptospiral growth inhibiting activity. All sera were negative in the MAT to *balcanica* at a minimum serum dilution of 1:12. Positive titre sera were used as controls.

Sera from a number of juvenile possums that were subjected to experimental challenge with *balcanica* were also investigated for growth inhibiting activity. The growth of leptospires in serum was scored on a subjective basis as 4, 3, 2 or 1. A score of 4 was equivalent to that of culture plus medium controls (approximately $1 \times 10^8$ organisms/ml). A score of 1 was equivalent to approximately 100 organisms per darkfield.

Experimental animal studies

Two females with pouched-young were selected from possums live-captured in September 1976 and maintained in captivity with their offspring until March, 1977. Selection was based on presence of serum titres and either dark-field microscopy or cultural evidence of leptospiruria. Serum and urine samples from dams and offspring were obtained at two month intervals. Sera were tested against *balcanica* antigen and urine samples were examined by dark-field microscopy and cultured by standard techniques. Kidneys from all animals were cultured at the termination of the experiment.

RESULTS

Prevalence of *balcanica* titres in possum populations inhabiting different ecosystems

Serological evidence of *balcanica* infection was found in possums inhabiting coastal seaface, deep forest and forest-pasture boundary

ecosystems. The prevalence of *balcanica* titres varied both within and between ecosystems (Table 8.2). The highest prevalence was found in possums inhabiting coastal cliffs at Makara and Pukerua Bay (77% and 74% in adult possums respectively). This environment was considered to be unfavourable for the survival of leptospires outside of the animal host. Soil samples revealed that there was an extremely high level of magnesium(>60 ppm extracting agent). Magnesium levels in coastal soils are closely associated with NaCl levels (J. Heslop, pers. comm.), thus indicating an environment subjected to high amounts of wind-borne salt water.

The prevalence of *balcanica* titres in adult possum populations inhabiting deep forest ecosystems varied from 0 to 45% (Table 8.2). No evidence of infection was found in the No. 1 sample from Maungataniwha. The population inhabiting this area was a high relative abundance. Only 1 of 27 adult possums was seropositive in the No 2. sample from Maungataniwha (4%). This was a female with a *balcanica* titre of 1:24.

Deep forest samples from Mangahao and Akatarawa had prevalences of *balcanica* titres in adult animals of 26% and 46% respectively. This difference was not statistically significant ($X^2$ = 1.68, P > 0.1).

Possum populations inhabiting forest-pasture boundary ecosystems at Pauatahanui and Dannevirke had similar serological prevalences of infection as those found in populations inhabiting pastoral locations (Chapter VI) and coastal seafaces (Table 8.2). There were significant differences however, between the prevalence of titres in adult possums from the forest-pasture boundary at Pakaututu and those from Pauatahanui and Dannevirke ($X^2$ = 7.54, P < 0.001; $X^2$ = 8.81, P < 0.001 respectively).

No titres were detected in the random sample of 100 sera from deep forest and coastal seaface ecosystems that were tested against *ballum*, *copenhageni*, *pomona* and *tarassovi* antigens.

Table 8.2 : Prevalence of *balcanica* titres in possum populations inhabiting different ecosystems

| Environmental description | Location | | No samples | No. sero-positive | Overall prevalence | Adult prevalence | Standard error of adult prevalence |
|---|---|---|---|---|---|---|---|
| Seaface | Pukurua Bay | | 38 | 22 | 58% | 77% | 7% |
| " | Makara | | 31 | 17 | 55% | 74% | 8% |
| Deep forest | Mangahao | | 14 | 5 | 36% | 45% | 13% |
| "    " | Maungataniwha | (1) | 47 | 0 | 0 | 0 | — |
| "    " | " | (2) | 49 | 1 | 2% | 4% | 3% |
| "    " | Akatarawa | (1) | 51 | 11 | 22% | 27% | 6% |
| "    " | " | (2) | 58 | 12 | 21% | 25% | 6% |
| Forest-Pasture boundary | Pakaututu | | 72 | 18 | 25% | 35% | 6% |
| " | Pauatahanui | | 50 | 24 | 48% | 68% | 7% |
| " | Dannevirke | | 70 | 35 | 50% | 67% | 6% |
| Farmland* | | | 154 | 66 | 43% | 70% | 4% |

* combined sample, from Chapter VI

The differences in age-specific prevalences of infection that were found in pastoral possum populations (Chapters IV and VI)were also found in populations inhabiting markedly different ecosystems (Tables 8.3 and 8.4). Only three of 109 juveniles had *balcanica* titres (3%) whereas 122 of 291 adults had titres (42%).

The geometric mean *balcanica* titres of seropositive adults from different locations are given in Table 8.5. (GMT's were not calculated for adults from Mangahao due to the low number of animals). The range of GMT's was from 1:76 to 1:148. Variation occurred between sexes within populations, and also between populations from different locations.

## Investigation of maternally-derived antibodies in sera from juvenile possums

The prevalence of *balcanica* titres and GMT's of positive sera in different weight-groups of pouched young and juvenile possums are given in Table 8.6. The growth curve of juvenile age-groups is given in Figure 8.2. (The serological prevalence was plotted against the mid-range value of each age group). Pouched-young aged less than 80 days had a prevalence of *balcanica* titres of 57%. The prevalence of maternally-derived titres in older age-groups declined gradually, however, 40% of juveniles aged between 181 and 190 days still had titres. Juveniles weighing between 900 g and 1500 g were not aged. It was estimated, from a continuation of the growth curve and a knowledge of the weights and ages at which juveniles reach sexual maturity (Chapter II), that juveniles weighing 1500 g would be approximately 250 days of age. The prevalence of titres in juveniles weighing between 1300 and 1500 g was 14%.

A scattergram of *balcanica* titres in pouched-young and juvenile possums of less than 190 days of age (Figure 8.3) demonstrated the lack of a significant association between the level of titres and age ($r = 0.22$, $P > 0.1$).

Table 8.3 : Prevalence of *balcanica* titres by sex and age-group in possums from different locations

| Location | | Males | | | Females | | | Trans. males | | | Trans. females | | | Juv. males | | | Juv. females | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | No. | No. sero. +ve | % | No. | No. sero. +ve | % | No. | No. sero. +ve | % | No. | No. sero. +ve | % | No. | No. sero. +ve | % | No. | No. sero. +ve | % |
| Pukerua Bay | | 11 | 9 | 82 | 15 | 11 | 73 | 4 | 2 | 50 | 2 | 0 | 0 | 3 | 0 | 0 | 3 | 0 | 0 |
| Makara | | 11 | 8 | 73 | 8 | 6 | 75 | 2 | 1 | 50 | 3 | 2 | 67 | 2 | 0 | 0 | 5 | 0 | 0 |
| Mangahao | | 6 | 2 | 33 | 5 | 3 | 60 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | – | – | 1 | 0 | 0 |
| Maungataniwha | (1) | 13 | 0 | 0 | 14 | 0 | 0 | 3 | 0 | 0 | 6 | 0 | 0 | 6 | 0 | 0 | 5 | 0 | 0 |
| | (2) | 15 | 0 | 0 | 12 | 1 | 8 | 5 | 0 | 0 | 4 | 0 | 0 | 6 | 0 | 0 | 7 | 0 | 0 |
| Akatarawa Forest | (1) | 14 | 4 | 29 | 16 | 4 | 25 | 5 | 1 | 20 | 4 | 1 | 25 | 5 | 0 | 0 | 7 | 0 | 0 |
| | (2) | 12 | 3 | 25 | 20 | 5 | 20 | 4 | 1 | 25 | 7 | 2 | 29 | 7 | 1 | 14 | 8 | 0 | 0 |
| Pakaututu | | 21 | 7 | 33 | 22 | 8 | 36 | 4 | 0 | 0 | 5 | 2 | 40 | 9 | 0 | 0 | 11 | 1 | 9 |
| Pauatahanui | | 13 | 8 | 62 | 15 | 11 | 73 | 5 | 2 | 40 | 3 | 2 | 67 | 8 | 1 | 13 | 6 | 0 | 0 |
| Dannevirke | | 27 | 18 | 67 | 21 | 14 | 67 | 7 | 2 | 29 | 5 | 1 | 20 | 6 | 0 | 0 | 4 | 0 | 0 |

Table 8.4 :  Prevalence of *balcanica* titres and geometric mean titres
in possums classified by sex and age-group

| Class | Number | No. sero positive | Prevalence% | GMT |
|-------|--------|-------------------|-------------|-----|
| Adult male | 143 | 59 | 41% | 1:100 |
| Adult female | 148 | 63 | 43% | 1:103 |
| Transitional-stage male | 40 | 9 | 23% | 1:129 |
| Transitional-stage female | 40 | 10 | 25% | 1:135 |
| Juvenile male | 52 | 2 | 4% | NT |
| Juvenile female | 57 | 1 | 2% | NT |

Table 8.5 :Geometric mean *balcanica* titres in adult males and adult
females from different locations

|  | Adult male | Adult female |
|---|---|---|
| Makara and Pukerua Bay | 1:132 | 1:103 |
| Akatarawa Forest (samples 1 and 2) | 1:86 | 1:76 |
| Pakaututu | 1:86 | 1:113 |
| Pauatahanui | 1:95 | 1:108 |
| Dannevirke | 1:93 | 1:123 |
| Farmland* | 1:148 | 1:99 |

* combined farmland sample, from Chapter VI.

GMT's of juveniles aged less than 190 days (900 g) showed a gradual decline with increasing age (Table 8.6). GMT's of sera from juveniles weighing between 900 and 1500 g showed little subsequent decline as weight increased.

The *balcanica* titres of dams were higher than those of their offspring in all cases.

Kidney cultures from all juveniles were negative.

## Growth inhibiting activity of sera from juvenile animals

Leptospiral growth inhibiting activity was detected in negative titre sera from two of ten juveniles weighing less than 900 g (20%). Negative titre sera from ten juveniles weighing more than 1200 g had no growth inhibiting activity (Table 8.7). Positive titre control sera all had growth inhibiting activity. Pre-inoculation negative titre sera from ten juveniles that were susceptible to experimental infection with *balcanica* all failed to inhibit the growth of leptospires. Preinoculation, negative titre sera from three of five juveniles that were insusceptible to experimental infection with *balcanica* (60%) had growth inhibiting activity.

The duration of the growth inhibting activity of sera from two juveniles in which maternally-derived antibody titres had fallen to undetectable levels is shown in Table 8.8. Growth inhibiting activity persisted in one female juvenile for six weeks and one male juvenile for four weeks.

It is shown in Chapter IX that the transient serological response to experimental *balcanica* infection of possum E2-5 (Chapter VII) was due to antibodies of the IgM class only. Subsequent, negative-titre serum samples taken from this animal at two week intervals had no growth inhibiting activity.

Table 8.6 : Prevalence of *balcanica* titres and GMT's of sera from possums from different age-groups*

| Weight (g) | Age (days) | No. tested | No. Seropositive | Prevalence | GMT of positive sera |
|---|---|---|---|---|---|
| < 40 | < 80 | 7 | 4 | 57% | 1:95 |
| 40 – 110 | 81 – 110 | 16 | 8 | 50% | 1:87 |
| 111 – 430 | 111 – 150 | 15 | 7 | 47% | 1:64 |
| 431 – 720 | 151 – 180 | 12 | 4 | 33% | 1:67 |
| 721 – 900 | 181 – 190 | 10 | 4 | 40% | 1:48 |
| 901 –1100 | – | 14 | 3 | 21% | 1:76 |
| 1101 –1300 | – | 16 | 2 | 13% | 1:48 |
| 1301 –1500 | – | 14 | 2 | 14% | 1:34 |

\* age determined from nomogram of Lyne and Verhagen (1957)
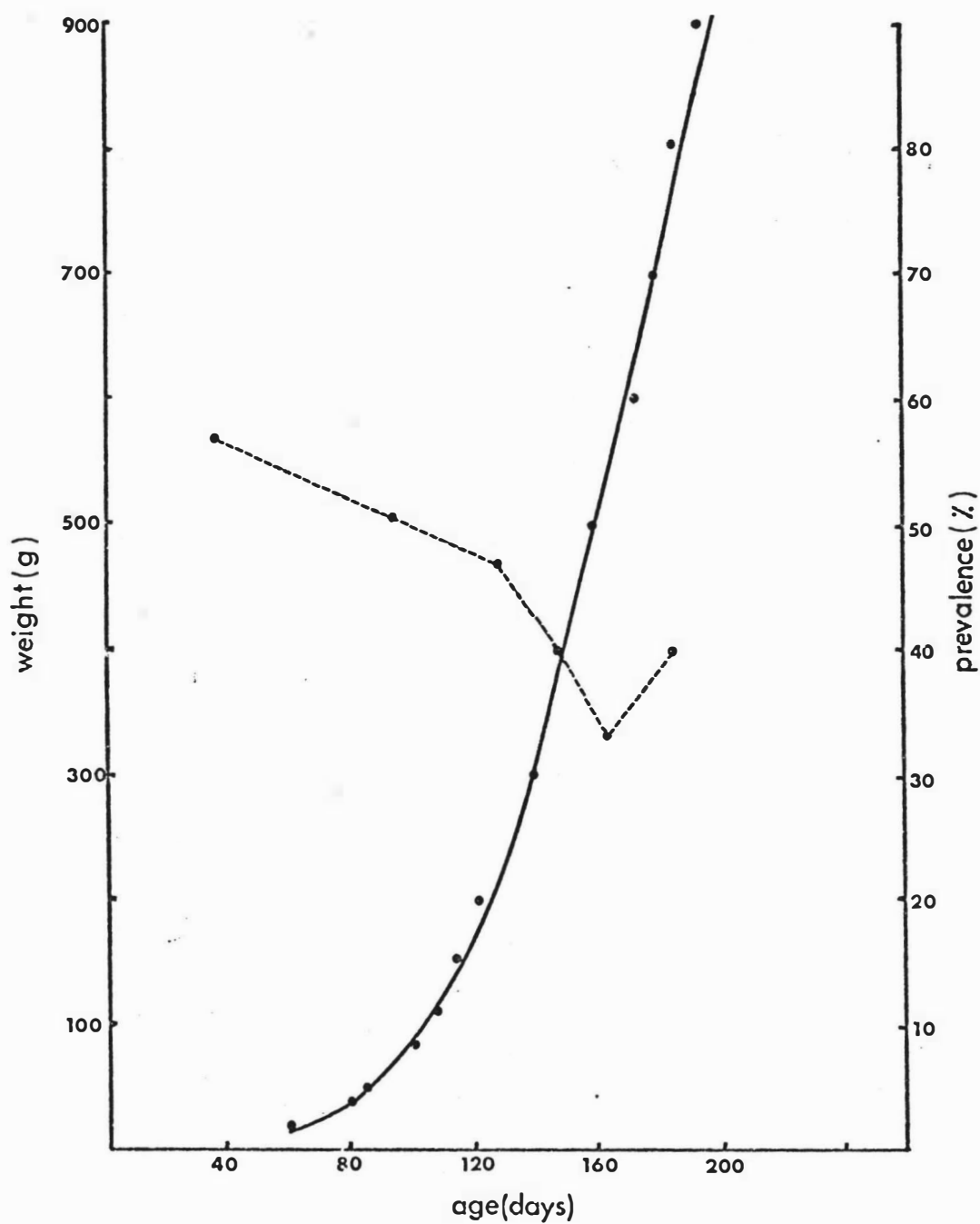
Figure 8.2. : Growth curve of juvenile possums*, and prevalence
of *balcanica* titres in different age-groups.

* growth curve calculated from the nomogram constructed
by Lyne and Verhagen (1957).

Figure 8.3. : *Balcanica* titres in juvenile possums of less than 190 days of age.
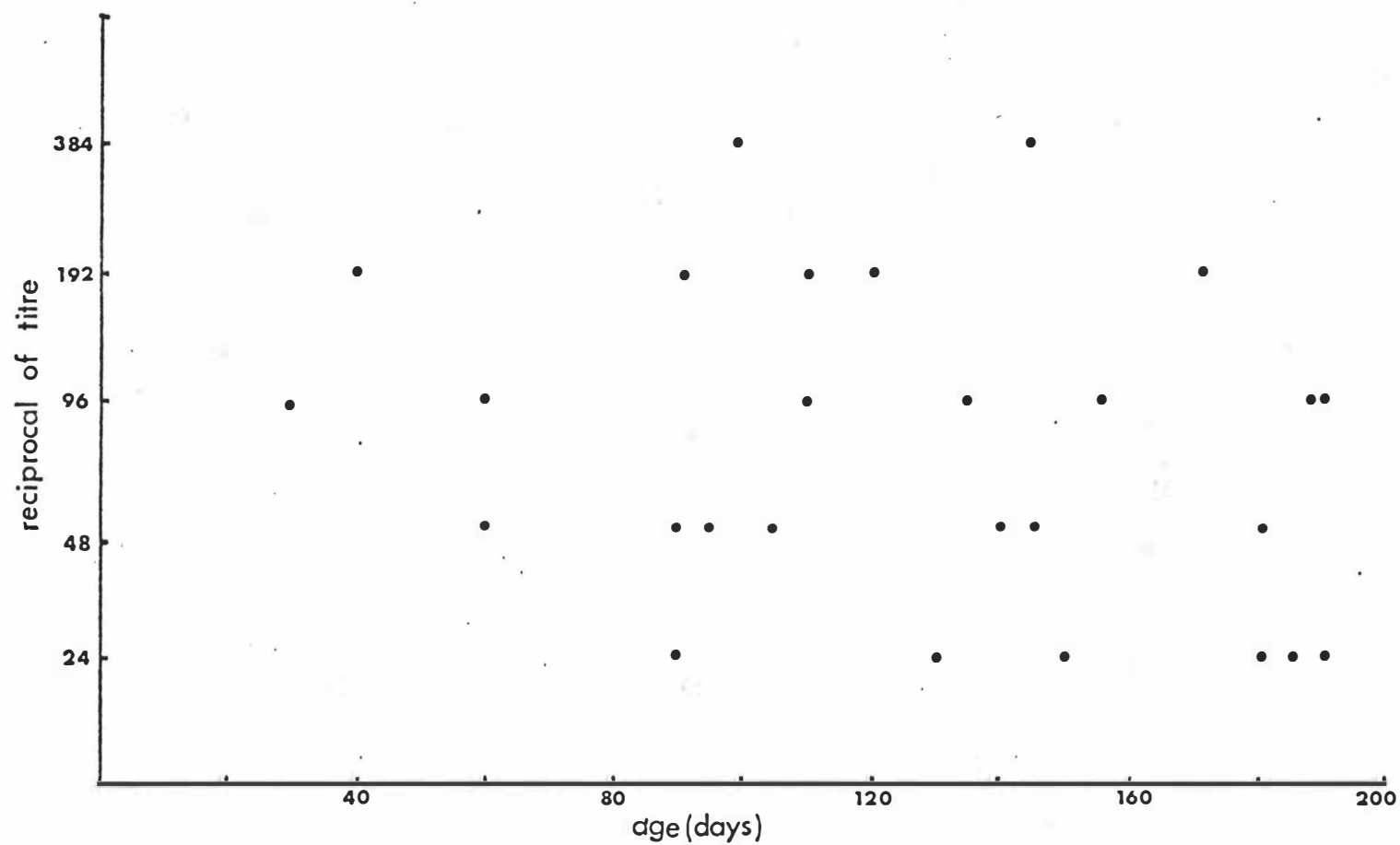
Experimental animal studies

Balcanica infection was not transmitted to juvenile possums
caged with leptospiruric dams. The serological and cultural results
from serial serum and urine samples from dams and joeys are given
in Table 8.9. Balcanica was recovered by kidney culture from one
dam at the termination of the experiment. Kidney cultures from
juveniles were negative.


DISCUSSION


The prevalence of balcanica infection in possums inhabiting
different ecosystems showed considerable variation , however there
was no apparent association between the different environmental
conditions in each ecosystem and the prevalence of infection in a
population. Of particular interest was the high prevalence of
infection in possums inhabiting coastal seafaces. Of the environments
investigated, these were considered to be the most unfavourable for
the survival of leptospires outside the host. In contrast, possums
inhabiting deep forest ecosystems had considerably lower prevalences
of infection, and the population at Maungataniwha was negative. These
environments were considered favourable for the survival of leptospires
outside the host.

It is apparent that the low pH of soil types that predominate
in New Zealand does not affect the prevalence of balcanica infection
in possums, or the prevalence of other serovars in maintenance host
species. Soil pH's ranged from 5.5 to 6.0 in this study, yet the
prevalence of infection was high in the majority of populations
sampled. Hellstrom (1978) demonstrated that pomona can survive for
at least 42 days in low pH (5.5) Manawatu soils. It is well
established that the in vitro survival of leptospires is affected
by the physical properties, moisture content and pH of soil
(Okazaki and Ringen, 1957; van der Hoeden, 1958). However, the
importance of the relative survival time outside the host in the
epidemiology of leptospirosis in a maintenance host population is

Table 8.7  Growth inhibiting activity of sera from juvenile possums

| No. | Wgt (g) | Suscepibility to exptl. infection | G.I. activity of sera — No. | prevalence | Score for leptospiral growth* |
|---|---|---|---|---|---|
| 10 | <900 | NT | 2 | 20% | 1, 2 |
| 10 | >1200 | NT | 0 | 0 | - |
| 10 | mixed | + | 0 | 0 | - |
| 5 | mixed | - | 3 | 60% | 1,2,2 |

* see text.  Score recorded for each serum with growth inhibiting activity.

Table 8.8.  Duration of growth inhibiting activity (GIA) in serial serum samples from two juvenile possums held in captivity.

| | | Age (days) | | | | |
|---|---|---|---|---|---|---|
| | | 180 * | 194 | 208 | 222 | 236 |
| Possum 1. | Titre[**] | 1:12 | 0 | 0 | 0 | 0 |
| | GIA(score) | +(1) | +(1) | +(2) | +(2) | - |

| | | Age (days) | | | | |
|---|---|---|---|---|---|---|
| | | 110* | 124 | 138 | 152 | 166 |
| Possum 2. | Titre | 1:12 | 0 | 0 | 0 | 0 |
| | GIA(score) | +(1) | +(2) | +(2) | - | - |

* Age at beginning of experiment calculated from nomogram of Lyne and Verhagen (1957)

** see text.

Table 8.9 : Serological and cultural results from adult female possums and joeys maintained in captivity for six months.

|          |   | September | | November | | January | | March | | |
|----------|---|-----------|-------------------|-----------|-------------------|-----------|-------------------|-----------|-------------------|------------------|
|          |   | titre | lepto-spiruria | titre | lepto-spiruria | titre | lepto-spiruria | titre | lepto-spiruia | kidney culture |
| Dam  | 1 | 1:192 | + | 1:192 | + | 1: 96 | + | 1:96 | + | + |
| Joey | 1 | 1: 24 | − | 1: 24 | − | 0 | − | 0 | − | − |
| Dam  | 2 | 1:768 | + | 1:384 | + | 1:192 | − | 1:192 | − | − |
| Joey | 2 | 0 | − | 0 | − | 0 | − | 0 | − | − |

not known.  Associations between soil characteristics and prevalence
of leptospiral infection in a particular species have been established
by some workers (Masaev, 1960; Twigg *et al*, 1969; Kingscote, 1970),
whereas others have been unable to establish such associations (Twigg
*et al*, 1968).  Soils at Makara and Pukerua Bay contained high
concentrations of NaCl, considered to be detrimental to the survival
of leptospires, yet these environments supported possum populations
with the highest recorded prevalences of *balcanica* infection.

Although considered unlikely,the absence of endemic
infection in possums from Maungataniwha might, in part, be associated
with particular environmental conditions affecting the survival of
leptospires outside of the host.  It was found that the forest-
pasture boundary sample from Pakaututu had a significnatly lower
prevalence of infection than samples from other forest-pasture
boundary ecosystems.  Pakaututu is located in the same geographical
area as Maungataniwha and the rhyolitic pumice soils of both
locations are derived from the same base material.

Differences in rainfall and vegetation in the various
ecosystems did not appear to be associated with differences in
prevalence of infection.  Rainfall would not be expected to be a
limiting factor in the environmental survival of leptospires in
the ecosystems under study, except perhaps on exposed and wind-
dessicated coastal seafaces.  High and similar prevalences of
infection were found in populations inhabiting ecosystems in which
the vegetation varied from virgin broadleaf-podocarp forest to
pasture grasses.

Subjective estimates of the relative abundance of all
populations sampled were either moderate or high.  If population
density was the only determinant of the prevalence of *balcanica*
infection in a population, it would be expected that all populations
sampled would have had a moderate or high prevalence of infection.
This was not the case in possums inhabiting deep forest ecosystems
at Maungataniwha and Akatarawa.  No endemic *balcanica* infection

occurred at Maungataniwha and the prevalence of infection in possums from Akatarawa was significantly lower than prevalences in pastoral and coastal seaface populations.

It can be concluded that the prevalence of *balcanica* infection in adult possums inhabiting different ecosystems has multiple determinants. There were considerably greater disparities in the prevalences of infection in the populations described in this Chapter than those occurring in pastoral populations investigated in Chapters IV and VI. Environmental factors affecting the survival of leptospires were not apparently associated with the epidemiology of the disease.

A generalisation drawn from the present epidemiological studies was that high prevalences of infection occurred in pasture and forest-pasture boundary populations of moderate or high population density, whereas the prevalence of infection in deep forest populations was considerably lower or absent. The lower prevalences of infection in forest possums were independent of population density. There are essential differences in the behaviour of possum populations inhabiting farmland and those inhabiting forest, and these have been reviewed in Chapter II. Populations inhabiting pastoral enviroments cover much greater areas than forest possums to utilise a wide variety of seasonally-available foods. Due to the limited availability of shelter on farmland, concentrations of animals tend to occur in small areas and nesting sites are more likely to be shared. It has also been found that the reproductive efficiency of pasture possums is higher than forest possums, and two breeding seasons may be recorded in some districts. In forest environments, only one breeding season occurs each year, and breeding success is often poor with high juvenile mortality rates. Some females do not breed until their fifth year. All these factors result in a higher degree of contact between individuals in pastoral populations that that which occurs in forest populations. If transmission of *balcanica* is by direct contact rather than via the environment,this may be the

explanation for the higher prevalence of infection found in pastoral possum populations than forest possum populations.

The absence of infection in the possum population at Maungataniwha does not seemingly support the hypothesis that population factors are a major determinant of the prevalence of *balcanica* infection in a population.

It must be considered, however, that *balcanica* may have never been introduced to possums inhabiting this area. The location from which possums were taken in the Waiau River Valley is geographically isolated by high ranges on both sides and is 20 km from the nearest forest-pasture boundary. The area is believed to have been colonised by the progeny of possums liberated at Lake Waikaremoana at the turn of the century (Pracy, 1962), which spread south through the Urewera National Park without contacting other populations. As such, the Maungataniwha population became established in isolation. There are several examples of geographically-isolated populations, especially on islands, being free of leptospirosis when the same species in other regions have endemic infection (Borg-Petersen and Fennestad, 1956; Twigg *et al*, 1968; McCaughey and Fairlie, 1971; Brockie, 1975).

A theoretical possibility for the lack of infection in possums from Maungataniwha was that of genetic resistance. This phenomenon has been described in a sub-species of *Apodemus agrarius* (Chernukha *et al*, 1975). This possibility was discounted by the successful experimental infection of two of three live-captured adults from Maungataniwha.

The differences in age-specific serological prevalence of *balcanica* infection that were found in pastoral possum populations were also found in possums inhabiting other ecosystems. The absence of infection in juvenile animals of an endemically-infected population indicates that they are either insusceptible (immune resistance) or are not challenged with the infectious agent until sexually mature.

The investigation of maternally-derived antibodies revealed that 40% of juveniles still had titres at 181 to 190 days of age.

The long duration of detectable levels of maternally-derived antibodies in juvenile possums is not seen in eutherian mammals. Kallai *et al* (1962) reported that titres persisted in laboratory rats for 32 days and Birnbaum *et al* (1974) found the detectable levels of maternally-derived antibodies in mice persisted for 70 days. All calves with maternally-derived *hardjo* titres in a study conducted by Hellstrom (1978) were sero-negative at 190 days and there was a significant regression of level of titre on age.

The persistence of significant levels of maternally-derived antibodies in juvenile possums for long periods may be a function of the physiology of this marsupial. Yadav (1971) demonstrated that immunoglobulins of the IgG class were absorbed across the gut wall for periods of up to 145 days of age. In eutherian mammals, the ability to absorb immunoglobulins from colostrum is lost within a few days of birth (Porter, 1972).

It is generally accepted that the presence of maternally-derived antibodies in the sera of juvenile animals protects them from infection and this was borne out by the experimental infection studies described in Chapter VII. Maternally-derived titres were still present in a small proportion of juveniles of 190 to 250 days of age. The very low prevalence of *balcanica* titres found in juvenile animals collected during epidemiological surveys were attributed to these persistent titres.

It has been demonstrated that negative titre sera taken from bovinesvaccinated with leptospiral bacterins four months previously contain leptospiral growth inhibiting activity (Tripathy *et al*, 1973; 1975). Growth inhibiting activity persisted in sera from juveniles, in which maternally-derived antibody titres had fallen to undetectable levels, for up to six weeks.

Growth inhibiting activity was also demonstrated in sera from three of five juveniles that were insusceptible to experimental infection, whereas sera from juveniles susceptible to experimental infection had no growth inhibiting activity. The growth inhibiting activity of sera therefore appears to extend, for limited periods, the protection afforded by maternal antibodies. Protection was due to antibodies of the IgG class (see fractionation of juvenile sera in Chapter IX), but was not afforded by antibodies of the IgM class that were produced following experimental infection in a juvenile possum.

Seronegative juvenile possums maintained in captivity with leptospiruric dams failed to become infected. This was in spite of the fact that maternally-derived antibody levels had decreased to non-detectable levels.

These findings provide the basis of an important concept in the epidemiology of *balcanica* infection in possums. Juvenile animals, fully susceptible to infection for several months after the loss of maternally-derived passive protection,do not become infected until the onset of sexual maturity. The absence of infection in sexually-immature animals has been described in a variety of maintenance host species (Ferris *et al*, 1961; Gordon-Smith *et al*, 1961c:Kallai *et al*, 1962; Wolf and Bohlander, 1965) and is also described in a laboratory mouse experimental model in Chapter XV. It is probable that the presence of infection in sexually mature animals is due to changes in behaviour, following the onset of sexual maturity, that facilitate transmission. This transmission may or may not be dependant on coitus. Venereal transmission of leptospirosis has been experimentally demonstrated in bovines (Sleight and Williams, 1961) and rabbits (Kiktenko *et al*, 1976) and many workers have suggested that the age-specific differences in prevalence of infection in maintenance host species are the result of transmission being dependent on the venereal route (Gordon - Smith *et al*, 1961c; Kallai *et al*, 1962; Trainer *et al*, 1963; Kemenes and Szeky, 1966; Twigg *et al*, 1968).

It is demonstrated in Chaptver XV however, that transmission in laboratory mice, although dependant on sexual maturity, is not dependant on coitus. *Ballum* was transmitted between female pairs of sexually-mature mice as well as between different-sex pairs of sexually-mature mice.

In a maintenance host population, the dependance of transmission on sexual maturity provides circumstantial evidence for the greater importance of transmission by direct contact rather than indirectly via the environment. If transmission is via the environment, it would be expected that fully susceptible, juvenile possums would have the same opportunity for becoming infected as sexually-mature animals. If transmission is dependant on behavioural changes that occur after the onset of sexual maturity, this would indicate that direct contact is probably the most important route of infection.

The hypothesis formulated from the investigations described in this chapter is that the most important mode of transmission of *balcanica* in possums is that of direct contact. Thus population dynamics will have a greater effect on the prevalence of *balcanica* infection in a population than environmental conditions favouring the survival of leptospires.

SUMMARY AND CONCLUSIONS

1.  *Balcanica* infection was endemic in possum populations inhabiting deep forest, forest-pasture boundary and coastal seaface ecosystems.

2.  The prevalence of infection did not appear to be associated with particular environmental conditions of different ecosystems.

3.  Populations inhabiting deep forest had a lower prevalence of infection than those inhabiting other ecosystems. This may be due to the particular population dynamics of deep forest populations.

4.  Endemic infection was not present in possums from Maungataniwha. The possibility that this was due to geographical isolation is discussed.

5.  Maternally-derived antibody titres were still present in 40% of sera from juveniles aged from 181 to 190 days.

6.  Leptospiral growth inhibiting activity was present in juvenile sera for up to six weeks after maternally-derived antibody titres had fallen to undetectable levels. This activity was associated with resistance to experimental infection.

7.  The fact that infection is confined to sexually-mature animals, even though juvenile animals in which passive protection has waned are fully susceptible to infection, is attributed to transmission being dependant on behavioural changes that follow the onset of sexual maturity.

8.  It is considered that the dependance of infection on sexual maturity provides circumstantial evidence of transmission by direct contact being more important than indirect environmental transmission in the maintenance of a high prevalence of infection.

CHAPTER IX

THE IMMUNOLOGICAL RESPONSE OF POSSUMS TO

LEPTOSPIRAL INFECTION

INTRODUCTION

The MAT is the test most frequently used to detect a sero-
logical response to leptospiral infection (Turner, 1968).
Agglutination tests can detect as little as 0.01 μg antibody nitrogen/
ml of serum, a sensitivity that compares favourably with that for
other serological tests (Barrett,1978).

In previous chapters,possums were shown to produce high levels
of agglutinins following *balcanica* infection.  This serological
response persisted for at least 13 months in experimentally-infected
animals.  It was also noted that a consistent paradoxical reaction
to *hardjo* occurred in possums infected with *balcanica*.

Although  the classes of immunoglobulins involved in the sero-
logical response to leptospiral infection in possums have not been
investigated previously, these have been characterised in other
species.  IgM (19S) antibody is generally the first immunoglobulin
to be synthesised (Pike *et al*,1965; Pike, 1967; Graves and Faine,
1970; Morris and Hussaini, 1974).  Synthesis of IgG (7S) antibody
follows, resulting in a mixed population of IgM and IgG antibodies
in serum.  A decline in the IgM concentration and an increase in IgG
results in the latter immunoglobulin being predominant in sera from
convalescent animals (Graves and Faine, 1970; Crawford, 1972; Chang
and Faine, 1974).

The temporal aspects of the transition from IgM to IgG have
been the subject of several investigations and results from different
species have been variable.  Crawford (1972) reported that following
an initial IgM response, IgG antibodies were first detected on day 23
post inoculation (p.i.) in sera from guinea pigs infected with
serovar *kennewicki*.  By day 40 p.i., 60% of the antibody activity was of
the IgG class.  Pike *et al*(1965) infected rabbits with *sejroe* and

found that 83% of antibody activity at day 44 p.i. was attributable
to IgG. In contrast, Hocker and Bauer (1965) repeatedly inoculated
rabbits with *biflexa* and found that the predominant antibody activity
remained in the IgM class. Graves and Faine (1970) also repeatedly
inoculated a rabbit with *biflexa*. At 216 days p.i., 33% of activity
was attributable to IgM.

Chernukcha *et al* (1976) conducted an extensive survey of humans
infected with a variety of serovars. Peak IgM and IgG antibody levels
were reached at four weeks and three weeks p.i. respectively. During
the first week of infection,the main antibody activity was of the IgM
class; but 8% of patients had IgG antibodies. Thus in some patients
IgM and IgG were synthesised simultaneously. These workers also
found a wide variation in the class of immunoglobulins contributing
to the serological response to leptospiral infection, with some
patients demonstrating only an IgM response and some demonstrating
only an IgG response.

Despite the disparities both within and between species, it is
apparent that the majority of antibody activity in sera in the early
stages of leptospiral infection is due to IgM. This immunoglobulin
is a more efficient agglutinating antibody than either IgG or IgA.
It has been reported that only 25 molecules of IgM are required for
50% erythrocyte agglutination, compared with more than 19000 IgG
molecules (Barrett, 1978). As time after infection increases, the
agglutinating activity of IgG antibody becomes an important part
of the serological response to infection. This class of antibody
appears to have a greater specificity than does IgM (Chang and Faine,
1974).

This chapter describes the immunoglobulin classes of antibodies
involved in the serological response of possums to infection with
*balcanica*. It also describes an investigation of the paradoxical
reaction to *hardjo* that is observed in possums infected with *balcanica*.
Experimentally-infected animals provided an opportunity to characterise
the long-term changes in antibody class and naturally-infected possums
and artificially-immunised rabbits provided comparative data. As
marsupials are a phyletic line separate from eutherian mammals, sera
from pouched-young and juveniles were also investigated to determine
the class of antibody involved in the passive transfer of immunoglobulins
from dam to offspring.

## MATERIALS AND METHODS

### Serum samples

Sera for fractionation were obtained from possums naturally and experimentally infected with *balcanica*. Six, randomly-selected sera from juveniles with maternally-derived agglutinins and six sera from naturally-infected adults were fractionated. The approximate age of juvenile possums (Table 9.1) was determined from the growth curve constructed by Lyne and Verhagen (1957).

Table 9.1 : Approximate age of juvenile possums from which serum was obtained for fractionation.

| Animal identification | Age (days) |
|---|---|
| J205 | 100 |
| J254 | 110 |
| J211 | 40 |
| J117 | 180 |
| J256 | 145 |

As field studies of possums were limited to cross-sectional sampling, the characterisation of the agglutination response to *balcanica* infection was carried out using sera from experimentally-infected animals. Representative samples for fractionation were obtained from the six possums that were infected in Experiment 4 of Chapter VII. These animals were monitored for thirteen months following inoculation. Sera were also fractionated from possums from Experiment 2 of Chapter VII. For comparative purposes, *balcanica* and *hardjo* antisera produced in rabbits were also fractionated.

### Column chromatography

Sera were fractionated by gel filtration using Sephadex G200[a]. The

[a]Pharmacia      Fine Chemicals AB, Uppsala, Sweden.

buffer used for elution (0.85% NaCl buffered to pH 7.4 with 0.05M phosphate buffer) contained 0.02% sodium azide. (Buffer preparation is described in Appendix V ). Fifteen grams of Sephadex G200 in one litre of buffer were swollen at $4^{\circ}$C for three days. The gel was transferred to a one litre measuring cylinder, mixed gently, and left to settle for 15 minutes. Fines were removed using a vacuum pump. The volume was made up to a litre and the procedure repeated. The swollen gel was then transferred to a side-arm flask, 100 mls of buffer added, and degassed under a vacuum of 700 mm Hg for ten minutes.

The column was poured using a slurry consisting of two parts of gel to one part of buffer. A reservoir[a] attached to the top of the 75 cm x 2.5 cm column enabled packing of the column to be achieved in one operation. Operating pressure during packing of the column was maintained at a constant pressure head of five cm of buffer.

One ml aliquots of sera were fractionated. 200 mg of sucrose were dissolved in each sample which was then layered on the top of the column. Columns were operated at room temperature under a constant pressure head ranging from 5 to 15 cm of buffer. The flow rate was maintained at approximately 20 mls/hour. The elution profile was monitored at an optical density of 280 nm and recorded graphically using an automated ISCO[b] system. Fractions of six mls volume were collected in an automated ISCO fraction collector.

## Serology

The dilution system used in the MAT to determine the agglutinating activity of fractions was a modification of that described for whole serum in Chapter III. 25 $\mu$l of saline were dispensed into all wells of a microtitre plate except the first row. The wells in the first row were filled with 50 $\mu$l of the fractions to be tested, and doubling dilutions were carried out down the plate using the 25 $\mu$l dilutor heads. As one ml of serum was fractionated and six ml fractions were collected, fractions were 1:6 dilutions. Addition of antigen produced a final

b    Golden Retriever Model, ISCO, P.O. Box 5347, Lincoln,
     Nebraska 68505, U.S.A.

fraction dilution of 1:12 in the first row of the microtitre plate,
1:24 in the second etc., down to 1:1536.

Fractions were randomised in microtitre plates so as to exclude
any bias in reading. All fractions,together with whole sera, were
tested against *balcanica* and *hardjo* simultaneously. Titres were
read as previously described although "half-titres" were also included
in the results (Table 9.2). The agglutinating activity of the
fractions was recorded as the reciprocal of the titre, rounded to
the nearest ten units. The agglutinating activity of all fractions
under each peak of the elution profile were aggregated and the
activities in peak I and peak II were expressed as a percentage of
total agglutinating activity. The ratio of the agglutinating
activity in peak I to that in peak II was also calculated.

## Immunoelectrophoresis (IEP)

IEP was used to analyse the distribution of immunoglobulins in
eluted fractions. Glass slides measuring 10 x 9 cm were layered
with 1% Ionagar[d] dissolved in 0.5% sodium barbital buffer, pH 8.6
(Appendix V ) containing 0.02% sodium azide. Plates were left at $4^{o}$C
for 24  hours to set. Troughs measuring 6 x 0.5 cm and wells with
a 0.5 cm diameter were cut at 1.0 cm centres in each plate. Agar
plugs were removed from the wells.

The wells were charged with antigen and each plate was subjected
to 100 v and 15 milliamps (10 volts/cm) for three hours. Agar was
then removed from the troughs which were then filled with appropriate
antiserum and the plates left at room temperature for 24 hours.
Precipitin lines were stained using amidoblack, followed by washing
with a solution of acetic acid, methanol and water (5 : 5 :1) for
three to seven days.

The antigens used consisted of pooled and concentrated fractions
from peaks I, II and III of the elution profiles of fractionated sera.
Pooled fractions from each peak were concentrated by dialysis against
polyethylene glycol (M.W.20,000). Rabbit anti-possum IgM and IgG

[d] Difco Laboratories, Detroit, Michigan, U.S.A.

Table 9.2 : Agglutinating acitivity of column chromatography
fractions rounded to the nearest ten units.

| Fraction dilution no. | MAT reading * | Agglutinating activity |
|---|---|---|
| | 0.5 | 10 |
| 1 | 1 | 10 |
| | 1.5 | 20 |
| 2 | 2 | 20 |
| | 2.5 | 30 |
| 3 | 3 | 50 |
| | 3.5 | 70 |
| 4 | 4 | 100 |
| | 4.5 | 130 |
| 5 | 5 | 190 |
| | 5.5 | 270 |
| 6 | 6 | 380 |
| | 6.5 | 540 |
| 7 | 7 | 770 |

\* end point 50% agglutination-lysis

antisera were kindly supplied by Mr P. Ramadass, Department of Veterinary Pathology and Public Health, Massey University.

## RESULTS

Gel filtration of possum sera on Sephadex G200 columns regularly resulted in a three peak elution profile. Peak I was contained in the first five to six fractions that were collected. Peak II was contained in the following eight to nine fractions. Peak III was contained in the last eight to nine fractions making up the elution profile. Anti-leptospiral IgM (19S) antibodies are contained in the first peak eluted on Sephadex G200 columns and IgG (7S) antibodies are contained in the second peak (Butler, 1969; Yadav, 1971; Duncan *et al*, 1972; Chang and Faine, 1974). To confirm the identity of the immunoglobulins contained in the fractions under each peak of the elution profile, fractions making up peaks I, II and III were pooled, concentrated and submitted to IEP (Plate 9.1.).

Precipitin lines produced by IEP of concentrated Peak I fractions and rabbit anti-possum IgM serum demonstrated that IgM was eluted in the first peak. No precipitin lines were formed when Peak I was run against rabbit anti-possum IgG, therefore no IgG was eluted in the first peak. A strong precipitin reaction was produced by IEP of concentrated peak II fractions and rabbit anti-possum IgG and this demonstrated that IgG was eluted in the second peak. There was also a slight precipitation reaction between concentrated Peak II fractions and rabbit anti-possum IgM. This was probably due to small amounts of monomeric IgM (M.W. 160 to 170,000) being eluted in the second peak. It is unlikely that this monomeric IgM contributed to the antibody activity of peak II fractions. Fractionated, high titre *balcanica* sera, in which high levels of IgM agglutinating activity were demonstrated, had no activity in IgG fractions (Tables 9.5 and 9.6). No precipitin lines were produced by concentrated peak III fractions run in IEP against rabbit anti-possum IgM or rabbit anti-possum IgG.

Although the IEP identification of immunoglobulin classes carried out in this study did not exclude the possibility of IgA being eluted in the second peak with IgG, it is unlikely that antibodies of this class would have contributed significantly to the agglutinating activity of Peak II fractions. IgA has been found to be present only in low

Plate 9.1:   Immunoelectrophoretic reaction of
             concentrated gel filtration fractions
             of possum serum with rabbit anti-possum IgM
             and IgG
             P1  =   peak I
             P2  =   peak II

             Anti IgM  =  rabbit anti-possum IgM
             Anti IgG  =  rabbit anti-possum IgG

P1

Anti IgM

P2

Anti IgG

P1

Anti IgM

concentrations in mammalian sera (Duncan *et al*, 1972; Barrett, 1978).

*Balcanica* agglutinins in sera from possums naturally infected with this serovar were found to be of both the IgM and IgG class (Table 9.3). IgM antibodies contributed from 28% to 53% of the agglutinating activity in different sera, whereas IgG antibodies contributed from 47 to 72%. *Hardjo* agglutinins in possums naturally infected with *balcanica* were also found in both the IgM and IgG classes. The agglutinating activity of *balcanica* and *hardjo* antibodies of the IgG class was similar. However the activity of *hardjo* antibodies of the IgM class was considerably higher than that of *balcanica* antibodies of the IgM class in the same sera.

Fractionation of serum from pouched-young and juvenile possums demonstrated that passive transfer of immunoglobulins from the dam was limited to antibodies of the 7S (IgG) class in all but one animal (Table 9.4). Joey 256 (145 days of age) had 8% of *balcanica* agglutinating activity in the IgM class. IgM antibody was not detected in fractionated sera of Joeys 254 and 211, both of which were younger than Joey 256.

*Balcanica* and *hardjo* IgG antibodies in fractionated sera from pouched-young and juvenile possums had similar agglutinating activity (Figure 9.1) and titres to these antigens in whole sera were within one serum dilution of each other. This was in contrast to the situation in *balcanica*-infected adult possums, where *hardjo* titres in whole sera were, in general, ≥ two serum dilutions higher than *balcanica* titres (Chapter VI and VII).

Serial serum samples from six possums experimentally infected with balcanica were fractionated and the class and relative agglutinating activity of *balcanica* and *hardjo* antibodies involved in the serological response were monitored for twelve months (Tables 9.5 to 9.11). Whole serum titres in these animals have been previously shown in Figure 7.8.

On day 15 p.i., *balcanica* and *hardjo* agglutinins were restricted to the IgM class (Table 9.5). Homologous *balcanica* agglutinins had considerably less activity than heterologous *hardjo* agglutinins. This

Table 9.3. : Distribution of *balcanica* and *hardjo* agglutinins in fractionated sera from adult possums.

| Fraction Number | Animal Identification | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | EID | | A123 | | 254 | | A116 | | W88 | |
| | B | H | B | H | B | H | B | H | B | H |
| I  1 | 10 | 30 | 10 | 20 | 20 | 100 | – | – | – | – |
| 2 | 30 | 190 | 30 | 100 | 50 | 380 | ·– | 10 | 10 | 20 |
| 3 | 100 | 380 | 50 | 190 | 100 | 770 | 20 | 50 | – | – |
| 4 | 50 | 380 | 50 | 100 | 20 | 190 | 10 | 10 | – | – |
| 5 | 50 | 100 | 20 | 20 | 20 | 100 | – | – | – | – |
| 6 | 20 | 50 | – | 10 | 20 | 100 | | | | |
| II  1 | 20 | 20 | 10 | 10 | 20 | 50 | – | – | – | – |
| 2 | 20 | 50 | 10 | 20 | 50 | 190 | 10 | 10 | – | – |
| 3 | 50 | 100 | 50 | 50 | 50 | 100 | 20 | 20 | 10 | 10 |
| 4 | 50 | 50 | 50 | 10 | 100 | 190 | 20 | 10 | 10 | 10 |
| 5 | 50 | 20 | 10 | 10 | 100 | 190 | 10 | 10 | – | – |
| 6 | 20 | 20 | 10 | – | 100 | 100 | – | 10 | – | – |
| 7 | 20 | 20 | – | – | 100 | 100 | – | – | – | – |
| 8 | 20 | 10 | – | – | 50 | 20 | – | – | – | – |
| 9 | 10 | 10 | | | 20 | 50 | | | | |
| III  1 | – | – | – | – | 20 | 20 | – | | – | – |
| 2 | – | – | – | – | 10 | 10 | – | | – | – |
| 3 | – | – | – | – | 10 | 10 | – | | – | – |
| 4 | – | – | – | – | – | – | – | | – | – |
| 5 | – | – | – | – | – | – | – | | – | – |
| 6 | – | – | – | – | – | – | – | | – | – |
| 7 | – | – | – | – | – | – | – | | – | – |
| 8 | – | – | – | | – | – | | | | |
| 9 | – | – | | | – | – | | | | |
| Whole serum | 380 | 1540 | 380 | 770 | 770 | 3070 | 50 | 100 | 20 | 50 |
| Peak I | 260 | 1130 | 160 | 440 | 230 | 1640 | 30 | 70 | 10 | 20 |
| Peak II | 260 | 300 | 140 | 100 | 590 | 990 | 60 | 60 | 20 | 40 |
| Total | 520 | 1430 | 300 | 540 | 820 | 2630 | 90 | 130 | 30 | 60 |
| % I | 50% | 79% | 53% | 81% | 28% | 62% | 33% | 54% | 33% | 33% |
| % II | 50% | 21% | 47% | 19% | 72% | 38% | 67% | 46% | 67% | 67% |
| I : II | 1 | 3.8 | 1.1 | 4.3 | 0.4 | 1.6 | 0.5 | 1.2 | 0.5 | 0.5 |

B = *balcanica*

H = *hardjo*

Table 9.4. : Distribution of *balcanica* and *hardjo* agglutinins in fractionated sera from juvenile possums.

| Fraction Number | | Animal Identification | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | J205 | | J254 | | J211 | | J117 | | J256 | |
| | | B | H | B | H | B | H | B | H | B | H |
| I | 1 | – | – | – | – | – | – | – | – | – | – |
| | 2 | – | – | – | – | – | – | – | – | – | – |
| | 3 | – | – | – | – | – | – | – | – | 20 | 20 |
| | 4 | – | – | – | – | – | – | – | – | – | – |
| | 5 | – | – | – | – | – | – | – | – | 20 | – |
| | 6 | – | – | | | – | – | – | – | | |
| II | 1 | 10 | 10 | 20 | 20 | 20 | 10 | 10 | – | 20 | 50 |
| | 2 | 20 | 20 | 20 | 20 | 50 | 50 | 30 | 20 | 100 | 100 |
| | 3 | 50 | 50 | 70 | 30 | 130 | 100 | 10 | – | 190 | 190 |
| | 4 | 100 | 70 | 50 | 50 | 100 | 130 | – | – | 100 | 100 |
| | 5 | 50 | 50 | 50 | 50 | 100 | 100 | – | – | 50 | 100 |
| | 6 | 30 | 20 | 20 | 20 | 50 | 50 | – | – | 20 | 20 |
| | 7 | 30 | 20 | 10 | – | 20 | 50 | – | – | – | 20 |
| | 8 | 10 | 10 | – | – | 10 | 10 | – | – | 10 | – |
| | 9 | – | – | – | – | 10 | – | – | – | | |
| III | 1 | – | – | – | – | – | – | – | – | 10 | 10 |
| | 2 | – | – | – | – | – | – | – | – | – | – |
| | 3 | – | – | – | – | – | – | – | – | | – |
| | 4 | – | – | – | – | – | – | – | – | – | – |
| | 5 | – | – | – | – | – | – | – | – | – | – |
| | 6 | – | – | – | – | – | – | – | – | – | – |
| | 7 | – | – | – | – | – | – | – | – | – | – |
| | 8 | | | | | | | – | – | – | – |
| | 9 | | | | | | | | | | |
| Whole serum | | 380 | 380 | 190 | 380 | 380 | 770 | 50 | 20 | 380 | 380 |
| Peak I | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 40 | 20 |
| Peak II | | 300 | 250 | 240 | 190 | 490 | 410 | 50 | 20 | 490 | 580 |
| Total | | 300 | 250 | 240 | 190 | 490 | 410 | 50 | 20 | 530 | 600 |
| % I | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 8% | 3% |
| % II | | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 92% | 97% |

B = *balcanica*

H = *hardjo*
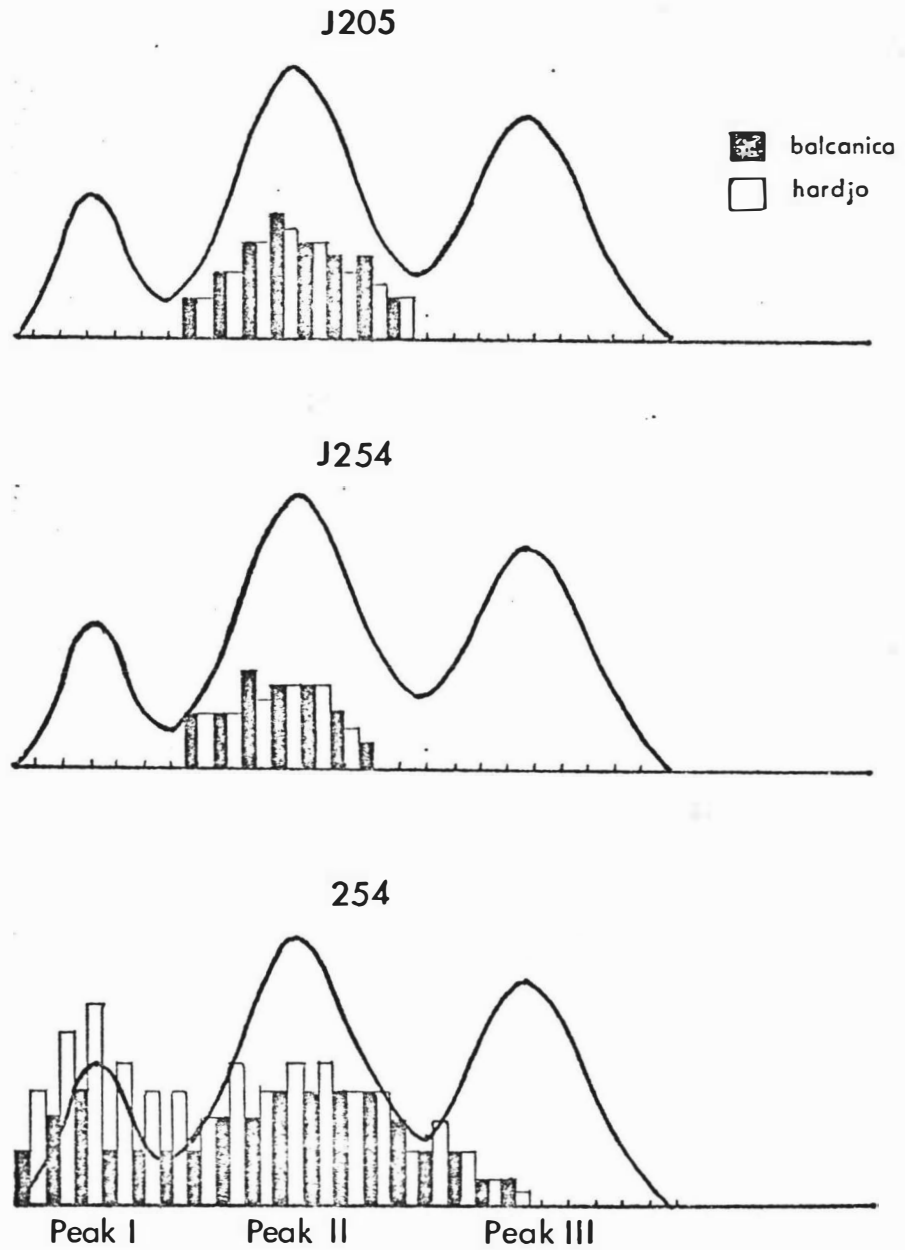
Figure 9.1. : Distribution of *balcanica* and *hardjo* agglutinins in
fractionated sera from Joey 205, Joey 254 and the
dam of Joey 254.

paradoxical reaction was seen in peak I fractions from all possum sera. Whole serum titres also showed a paradoxical reaction to *hardjo*. *Hardjo* titres were from two to four serum dilutions higher than *balcanica* titres for the same serum.

Similar results were observed on day 28 p.i. (Table 9.6). *Balcanica* and *hardjo* agglutinins were restricted to the IgM class and a paradoxical *hardjo* reaction was present in peak I fractions and whole sera.

Synthesis of antibodies of the IgG class was first detected on day 60 p.i. (Table 9.7). Agglutinating activity was present in Peak II of the elution profile of fractionated serum from three of six possums (50%). 18 to 33% of the *balcanica* agglutinating activity at this time was due to IgG. A paradoxical reaction to *hardjo* occurred only in fractions containing IgM antibody. This resulted in *hardjo* antibodies of the IgG class contributing only 8 to 11% of the total agglutinating activity for *hardjo*. The trend of a higher percentage of *balcanica* than *hardjo* agglutinating activity in the same serum sample residing in the IgG class was continued for the duration of the experiment.

By day 120 p.i., IgG antibody was present in fractionated serum from all possums (Table 9.8). From 50 to 62% of *balcanica* agglutinating activity was attributable to IgG. *Balcanica* and *hardjo* agglutinins of the IgG class had similar activity. *Hardjo* antibodies of the IgM class had greater activity than did *balcanica* IgM agglutinins.

This trend was continued in fractionated sera collected on days 210 and 270 p.i. (Tables 9.9 and 9.10). By day 360 p.i., from 56 to 89% of *balcanica* agglutinating activity was attributable to IgG; 11 to 44% being attributable to IgM. Due to the paradoxical reaction still apparent in *hardjo* agglutinins of the IgM class, the percentage of *hardjo* agglutinating activity attributable to IgG (44% to 81%) was lower than that for *balcanica*.

The transition of *balcanica* and *hardjo* agglutinins from the IgM to the IgG class with increasing time after inoculation is

Table 9.5. : Distribution of *balcanica* and *hardjo* agglutinins on day 15 p.i. in fractionated sera from possums experimentally infected with *balcanica*.

| Fraction Number | Animal Identification | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E4-2 | | E4-4 | | E4-5 | | E4-6 | | E4-7 | | E4-8 | |
| | B | H | B | H | B | H | B | H | B | H | B | H |
| I 1 | – | 10 | 20 | 70 | 10 | 20 | 10 | 20 | 20 | 30 | 10 | 10 |
| 2 | 10 | 30 | 50 | 190 | 50 | 100 | 130 | 380 | 50 | 190 | 50 | 50 |
| 3 | 50 | 100 | 130 | 380 | 100 | 380 | 270 | 1540 | 130 | 380 | 100 | 190 |
| 4 | 70 | 190 | 380 | 770 | 50 | 380 | 380 | 770 | 70 | 190 | 50 | 70 |
| 5 | 50 | 50 | 100 | 190 | 20 | 100 | 130 | 770 | 20 | 50 | 30 | 100 |
| 6 | 10 | 20 | 20 | 100 | – | 20 | 30 | 190 | | | – | 30 |
| II 1 | – | – | – | 20* | – | – | 30* | 50* | – | – | – | – |
| 2 | – | – | – | 10 | – | – | – | – | – | – | – | – |
| 3 | – | – | – | – | – | – | – | – | – | – | – | – |
| 4 | – | – | – | 10 | – | – | – | – | – | – | – | – |
| 5 | – | – | – | 10 | – | – | – | – | – | – | – | – |
| 6 | – | – | – | – | – | – | – | – | – | – | – | – |
| 7 | – | – | – | – | – | – | – | – | – | – | – | – |
| 8 | – | – | – | – | – | – | – | – | – | – | – | – |
| 9 | | | – | | – | – | | | – | | | |
| III 1 | – | – | – | | – | – | – | – | – | – | – | – |
| 2 | – | – | – | | – | – | – | – | – | – | – | – |
| 3 | – | – | | 10 | – | – | 10 | – | – | – | – | – |
| 4 | 10 | 20 | | – | – | – | – | – | 10 | 20 | – | – |
| 5 | – | – | – | – | – | – | – | – | – | – | – | – |
| 6 | – | – | – | – | – | – | – | – | – | – | – | – |
| 7 | – | – | – | – | – | – | – | – | – | – | – | – |
| 8 | | | – | | – | – | – | – | – | – | – | – |
| 9 | | | | | | | | | – | – | | |
| Whole serum | 190 | 770 | 770 | 6140 | 190 | 1540 | 770 | 12290 | 190 | 1540 | 100 | 770 |
| Peak I | 190 | 400 | 700 | 1700 | 230 | 960 | 950 | 3670 | 290 | 840 | 240 | 450 |
| Peak II | – | – | – | 20 | – | – | – | – | – | – | – | – |
| Total | 190 | 400 | 700 | 1720 | 230 | 960 | 950 | 3670 | 290 | 840 | 240 | 450 |
| % I | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% |
| % II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| I : II | – | – | – | – | – | – | – | – | – | – | – | – |

B = *balcanica*

H = *hardjo*

\* tail activity from Peak I not included in analysis

Table 9.6. : Distribution of *balcanica* and *hardjo* agglutinins on day 28 p.i. in fractionated sera from possums experimentally infected with *balcanica*.

| Fraction Number | Animal Identification | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E4-2 | | E4-4 | | E4-5 | | E4-6 | | E4-7 | | E4-8 | |
| | B | H | B | H | B | H | B | H | B | H | B | H |
| I 1 | - | 20 | - | 50 | - | 20 | 30 | 100 | NT | NT | - | - |
| 2 | 50 | 100 | 50 | 190 | 20 | 50 | 100 | 380 | " | " | 10 | 50 |
| 3 | 100 | 380 | 70 | 380 | 30 | 100 | 100 | 770 | " | " | 20 | 70 |
| 4 | 20 | 100 | 10 | 380 | 20 | 70 | 50 | 190 | " | " | 20 | 50 |
| 5 | 10 | 30 | 10 | 100 | - | 20 | 30 | 190 | " | " | 10 | 20 |
| 6 | - | 10 | | | | | - | 30 | | | | |
| II 1 | - | - | - | 20* | - | - | - | - | " | " | - | - |
| 2 | - | - | - | - | - | - | - | - | " | " | - | - |
| 3 | - | - | - | - | - | - | - | - | " | " | - | - |
| 4 | - | - | - | - | - | - | - | - | " | " | - | - |
| 5 | - | - | - | - | - | - | - | - | " | " | - | - |
| 6 | - | - | - | - | - | - | - | - | " | " | - | - |
| 7 | - | - | - | - | - | - | - | - | " | " | - | - |
| 8 | - | - | - | - | | | - | - | " | " | - | - |
| 9 | - | - | | | | | | | | | | |
| III 1 | - | - | - | - | - | - | - | - | " | " | - | - |
| 2 | - | - | - | - | - | - | - | - | " | " | - | - |
| 3 | - | - | - | - | - | - | - | - | " | " | - | - |
| 4 | - | - | - | - | - | - | - | - | " | " | - | - |
| 5 | - | - | - | - | - | - | - | - | " | " | - | - |
| 6 | - | - | - | - | - | - | - | - | " | " | - | - |
| 7 | - | - | - | - | | | - | - | " | " | - | - |
| 8 | - | - | - | - | | | - | - | | | - | - |
| 9 | - | - | | | | | | | | | | |
| Whole serum | 100 | 770 | 190 | 1540 | 50 | 190 | 190 | 1540 | 170 | 770 | 100 | 380 |
| Peak I | 180 | 640 | 140 | 1100 | 70 | 260 | 310 | 1660 | NT | NT | 60 | 190 |
| Peak II | - | - | - | - | - | - | - | | " | " | - | - |
| Total | 180 | 640 | 140 | 1100 | 70 | 260 | 310 | 1660 | " | " | 60 | 190 |
| % I | 100% | 100% | 100% | 100% | 100% | 100% | 100% | 100% | " | " | 100% | 100% |
| % II | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | " | " | 0 | 0 |
| I : II | - | - | - | - | - | - | - | - | " | " | - | - |

B = *balcanica*      * tail activity from Peak I not included in analysis.

H = *hardjo*

Table 9.7. : Distribution of *balcanica* and *hardjo* agglutinins on day 60 p.i. in fractionated sera from possums experimentally infected with *balcanica*.

| Fraction Number | Animal Identification | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E4-2 | | E4-4 | | E4-5 | | E4-6 | | E4-7 | | E4-8 | |
| | B | H | B | H | B | H | B | H | B | H | B | H |
| I 1 | – | 20 | – | – | – | 10 | – | 50 | – | 20 | – | – |
| 2 | 10 | 100 | 10 | 50 | 10 | 30 | 20 | 100 | 20 | 100 | 10 | 20 |
| 3 | 50 | 190 | 30 | 100 | 20 | 100 | 50 | 380 | 70 | 130 | 30 | 50 |
| 4 | 50 | 130 | 20 | 130 | 10 | 50 | 20 | 190 | 50 | 50 | 20 | 30 |
| 5 | 10 | 100 | – | 70 | 10 | 10 | – | 100 | 10 | 10 | – | – |
| 6 | – | 30 | – | 20 | | | – | 30 | | | | |
| II 1 | – | – | – | – | – | – | 10 | 10 | – | – | – | – |
| 2 | – | – | 10 | – | – | – | – | 10 | 10 | 10 | 10 | 10 |
| 3 | – | – | – | – | – | – | 10 | 50 | 10 | 20 | 10 | – |
| 4 | – | – | – | – | – | – | – | – | – | 10 | 10 | – |
| 5 | – | – | – | – | – | – | – | – | 10 | – | – | – |
| 6 | – | – | – | – | – | – | – | – | – | – | – | – |
| 7 | – | – | – | – | – | – | – | – | – | – | | |
| 8 | – | – | – | – | | | – | – | | | | |
| 9 | – | – | | | | | | | | | | |
| III 1 | – | – | – | – | – | – | – | – | – | – | | |
| 2 | – | – | – | – | – | – | – | – | – | – | | |
| 3 | – | – | – | – | – | – | – | – | – | – | | |
| 4 | – | – | – | – | – | – | – | – | – | – | | |
| 5 | – | – | – | – | – | – | – | – | – | – | | |
| 6 | – | – | – | – | – | – | – | – | – | – | | |
| 7 | – | – | – | – | – | – | – | – | – | – | | |
| 8 | – | – | – | – | | | – | – | | | | |
| 9 | – | – | | | | | | | | | | |
| Whole Serum | 190 | 1540 | 100 | 380 | 50 | 100 | 190 | 770 | 190 | 380 | 100 | 190 |
| Peak I | 120 | 570 | 60 | 370 | 50 | 200 | 90 | 850 | 150 | 310 | 60 | 100 |
| Peak II | – | – | 10 | 10 | – | – | 20 | 70 | 30 | 40 | 30 | 10 |
| Total | 120 | 570 | 70 | 370 | 50 | 200 | 110 | 920 | 180 | 350 | 90 | 110 |
| % I | 100% | 100% | 86% | 100% | 100% | 100% | 82% | 92% | 83% | 89% | 67% | 91% |
| % II | 0 | 0 | 14% | 0 | 0 | 0 | 18% | 8% | 17% | 11% | 33% | 9% |
| I : II | – | – | 6 | – | – | – | 4.6 | 11.5 | 4.9 | 8.1 | 2 | 10 |

Table 9.8. : Distribution of *balcanica* and *hardjo* agglutinins on day 120 p.i. in fractionated sera from possums experimentally infected with *balcanica*.

| Fraction Number | Animal Identification | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E4-2 | | E4-4 | | E4-5 | | E4-6 | | E4-7 | | E4-8 | |
| | B | H | B | H | B | H | B | H | B | H | B | H |
| I 1 | – | – | – | 30 | NT | NT | – | 10 | 10 | 10 | – | 10 |
| 2 | 10 | 20 | 20 | 20 | " | " | 10 | 50 | 20 | 50 | 20 | 20 |
| 3 | 20 | 50 | 50 | 190 | " | " | 50 | 100 | 10 | 50 | 20 | 50 |
| 4 | 20 | 50 | 30 | 100 | " | " | 20 | 50 | 10 | 30 | 10 | 30 |
| 5 | – | 50 | 10 | 50 | " | " | 20 | 30 | – | 10 | 10 | 20 |
| 6 | 10 | 20 | – | 20 | " | " | – | 10 | – | – | – | 10 |
| II 1 | – | 10 | – | – | " | " | – | 10 | 10 | – | 10 | – |
| 2 | 10 | 10 | 10 | 20 | " | " | 10 | 20 | 10 | 10 | 20 | – |
| 3 | 10 | 20 | 20 | 20 | " | " | 20 | 30 | 10 | 10 | 20 | 10 |
| 4 | 20 | 30 | 50 | 50 | " | " | 20 | 50 | 20 | 20 | 10 | 20 |
| 5 | 20 | 20 | 30 | 50 | " | " | 20 | 20 | 10 | 10 | 10 | 20 |
| 6 | 10 | 10 | 20 | 20 | " | " | 10 | 20 | 10 | – | 10 | 10 |
| 7 | – | – | 10 | 10 | " | " | 10 | 10 | – | – | 10 | – |
| 8 | – | – | 10 | – | " | " | 10 | – | – | – | 10 | – |
| 9 | | | – | – | " | " | – | – | – | – | | |
| III 1 | – | – | 10 | – | " | " | – | – | – | – | – | – |
| 2 | – | – | – | – | " | " | – | – | – | – | – | – |
| 3 | – | – | – | – | " | " | – | – | – | – | – | – |
| 4 | – | – | – | – | " | " | – | – | – | – | – | – |
| 5 | – | – | – | – | " | " | – | – | – | – | – | – |
| 6 | – | – | – | – | " | " | – | – | – | – | – | – |
| 7 | – | – | – | – | " | " | – | – | – | – | – | – |
| 8 | – | – | – | – | " | " | – | – | – | – | – | – |
| 9 | | | | | " | " | | | – | – | | |
| Whole serum | 100 | 380 | 100 | 770 | NT | NT | 190 | 770 | 190 | 190 | 100 | 380 |
| Peak I | 60 | 190 | 110 | 410 | " | " | 100 | 250 | 50 | 150 | 60 | 140 |
| Peak II | 70 | 100 | 150 | 170 | " | " | 100 | 160 | 70 | 50 | 100 | 60 |
| Total | 130 | 290 | 260 | 580 | " | " | 200 | 410 | 120 | 200 | 160 | 200 |
| % I | 46% | 66% | 42% | 71% | " | " | 50% | 61% | 42% | 75% | 38% | 70% |
| % II | 54% | 34% | 58% | 29% | " | " | 50% | 39% | 58% | 25% | 62% | 30% |
| I : II | 0.9 | 1.9 | 0.9 | 2.5 | " | " | 1 | 1.6 | 0.7 | 3 | 0.6 | 2.3 |

B = *balcanica*

H = *hardjo*

Table 9.9. : Distribution of *balcanica* and *hardjo* agglutinins on
day 210 p.i. in fractionated sera from possums
experimentally infected with *balcanica*.

| Fraction Number | Animal Identification | | | | | | | | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | E4-2 | | E4-4 | | E4-5 | | E4-6 | | E4-7 | | E4-8 | |
| | B | H | B | H | B | H | B | H | B | H | B | H |
| I 1 | – | 10 | 10 | 10 | – | 10 | – | 20 | – | 10 | – | – |
| 2 | 30 | 50 | 10 | 20 | 10 | 30 | 30 | 100 | 10 | 20 | 20 | 50 |
| 3 | 20 | 50 | 10 | 20 | 10 | 50 | 30 | 100 | 20 | 50 | 20 | 100 |
| 4 | 20 | 30 | 10 | 20 | 10 | 20 | 20 | 50 | 10 | 50 | 10 | 50 |
| 5 | 20 | 20 | – | 10 | – | 10 | – | 10 | – | 20 | – | 10 |
| 6 | – | – | | | | | | | – | 10 | – | – |
| II 1 | 10 | 10 | 10 | 10 | – | – | – | 10 | – | – | – | – |
| 2 | 20 | 10 | 10 | 20 | – | – | 50 | 50 | 10 | – | – | 10 |
| 3 | 30 | 20 | 10 | 10 | 10 | 10 | 50 | 100 | 10 | 10 | 50 | 30 |
| 4 | 50 | 30 | 20 | 20 | 10 | 20 | 130 | 100 | 20 | 50 | 100 | 50 |
| 5 | 20 | 10 | 20 | 20 | 10 | 20 | 70 | 50 | – | 10 | 20 | 20 |
| 6 | 20 | 10 | 10 | 10 | 20 | 20 | 20 | 20 | 10 | 10 | 10 | 10 |
| 7 | – | – | 10 | 10 | 10 | 10 | 30 | – | – | – | – | – |
| 8 | – | – | – | – | – | 10 | | | | | – | – |
| 9 | – | – | | | | | | | | | – | – |
| III 1 | – | – | – | – | – | – | – | – | – | – | – | – |
| 2 | – | – | – | – | – | – | – | – | – | – | – | – |
| 3 | – | – | – | – | – | – | – | – | – | – | – | – |
| 4 | – | – | – | – | – | – | – | – | – | – | – | – |
| 5 | – | – | – | – | – | – | – | – | – | – | – | – |
| 6 | – | – | – | – | – | – | – | – | – | – | – | – |
| 7 | – | – | – | – | – | – | – | – | – | – | – | – |
| 8 | – | – | – | – | – | – | | | – | – | – | – |
| 9 | | | | | | | | | | | – | – |
| Whole serum | 190 | 380 | 190 | 380 | 100 | 380 | 380 | 380 | 100 | 380 | 100 | 380 |
| Peak I | 90 | 160 | 40 | 80 | 30 | 120 | 80 | 280 | 40 | 160 | 50 | 210 |
| PeakII | 150 | 90 | 90 | 100 | 60 | 90 | 350 | 330 | 50 | 80 | 180 | 120 |
| Total | 240 | 250 | 130 | 180 | 90 | 210 | 430 | 610 | 90 | 240 | 230 | 330 |
| % I | 38% | 64% | 31% | 44% | 33% | 57% | 19% | 46% | 44% | 67% | 22% | 64% |
| % II | 62% | 36% | 69% | 56% | 67% | 43% | 81% | 54% | 56% | 33% | 78% | 36% |
| I:II | 0.6 | 1.8 | 0.5 | 0.8 | 0.5 | 1.3 | 0.2 | 0.9 | 0.8 | 2 | 0.3 | 1.8 |

B = *balcanica*

H = *hardjo*

Table 9.10. : Distribution of *balcanica* and *hardjo* agglutinins on day 270 p.i. in fractionated sera from possums experimentally infected with *balcanica*.

| Fraction Number | Animal Identification | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | E4-2 | | E4-4 | | E4-5 | | E4-6 | | E4-7 | | E4-8 | |
| | B | H | B | H | B | H | B | H | B | H | B | H |
| I 1 | 10 | – | – | – | 20 | 10 | – | 30 | – | 10 | – | – |
| 2 | – | 20 | 10 | 50 | 20 | 50 | 20 | 50 | 10 | 50 | – | 10 |
| 3 | 20 | 70 | 20 | 100 | 20 | 30 | 10 | 30 | 10 | 30 | 10 | 20 |
| 4 | 20 | 50 | 10 | 20 | – | 10 | – | 10 | 10 | 10 | 10 | 20 |
| 5 | – | 20 | – | 10 | | | | | – | – | 10 | 10 |
| 6 | | | | | | | | | | | | |
| II 1 | – | – | 20 | 10 | 10 | 10 | 50 | 20 | – | – | 50 | 20 |
| 2 | 10 | 20 | 10 | 10 | 20 | 20 | 130 | 100 | 10 | 10 | 50 | 20 |
| 3 | 50 | 50 | 100 | 50 | 20 | 20 | 50 | 20 | 20 | 10 | 10 | 10 |
| 4 | 50 | 50 | 50 | 30 | – | 20 | 50 | 10 | 10 | 10 | – | – |
| 5 | 20 | 30 | 10 | 10 | 30 | 20 | 10 | 10 | 10 | 10 | – | – |
| 6 | 20 | – | – | – | 30 | 10 | | | – | – | – | – |
| 7 | 10 | 10 | | | 10 | 10 | | | | | | |
| 8 | | | | | | | | | | | | |
| 9 | | | | | | | | | | | | |
| III 1 | – | – | – | – | – | – | – | – | – | – | – | – |
| 2 | – | – | – | – | – | – | – | – | – | – | – | – |
| 3 | – | – | – | – | – | – | – | – | – | – | – | – |
| 4 | – | – | – | – | – | – | – | – | – | – | – | – |
| 5 | – | – | – | – | – | – | – | – | – | – | – | – |
| 6 | – | – | – | – | – | – | | | – | – | – | – |
| 7 | – | – | – | – | – | – | | | – | – | | |
| 8 | – | – | | | – | – | | | | | | |
| 9 | | | | | | | | | | | | |
| Whole serum | 100 | 390 | 100 | 390 | 50 | 190 | 190 | 190 | 50 | 380 | 190 | 190 |
| Peak I | 50 | 160 | 40 | 180 | 60 | 100 | 30 | 120 | 30 | 100 | 30 | 60 |
| Peak II | 160 | 160 | 190 | 110 | 120 | 110 | 290 | 160 | 50 | 40 | 110 | 50 |
| Total | 210 | 320 | 230 | 290 | 180 | 210 | 320 | 280 | 80 | 140 | 140 | 110 |
| % I | 24% | 50% | 17% | 62% | 33% | 48% | 9% | 43% | 33% | 71% | 21% | 55% |
| % II | 76% | 50% | 83% | 38% | 67% | 52% | 91% | 57% | 62% | 29% | 79% | 45% |
| I : II | 0.3 | 1 | 0.2 | 1.6 | 0.5 | 0.9 | 0.1 | 0.8 | 0.6 | 2.4 | 0.3 | 1.2 |

B = *balcanica*

H = *hardjo*

Table 9.11. : Distribution of *balcanica* and *hardjo* agglutinins on day 360 p.i. in fractionated sera from possums experimentally infected with *balcanica*.

Animal Identification

| Fraction Number | E4-2 B | E4-2 H | E4-4 B | E4-4 H | E4-5 B | E4-5 H | E4-6 B | E4-6 H | E4-7 B | E4-7 H | E4-8 B | E4-8 H |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| I  1 | – | – | NT | NT | – | – | 10 | 30 | – | – | – | – |
| 2 | 10 | 20 | " | " | – | 10 | 20 | 50 | 10 | 30 | 10 | 20 |
| 3 | 20 | 50 | " | " | 10 | 30 | 10 | 30 | 20 | 50 | 10 | 20 |
| 4 | 20 | 50 | " | " | 10 | 20 | – | 10 | 10 | 10 | – | 10 |
| 5 | – | 20 | " | " | 10 | 10 | – | – | – | – | – | – |
| 6 | – | – | " | " | | | | | | | | |
| II  1 | 10 | – | " | " | – | 10 | 20 | 20 | 10 | 10 | – | 20 |
| 2 | 10 | 10 | " | " | 50 | 30 | 50 | 30 | 10 | 20 | 50 | 100 |
| 3 | 20 | 20 | " | " | 50 | 30 | 50 | 50 | 20 | 20 | 50 | 70 |
| 4 | 30 | 20 | " | " | 20 | 50 | 10 | 20 | 10 | 20 | 30 | 20 |
| 5 | 30 | 30 | " | " | 50 | 20 | 10 | 10 | – | 10 | 20 | 10 |
| 6 | 10 | 10 | " | " | 20 | 10 | 10 | – | – | – | 10 | – |
| 7 | 10 | 20 | " | " | – | – | – | – | – | – | – | – |
| 8 | – | – | " | " | | | | | | | | |
| 9 | | | " | " | | | | | | | | |
| III  1 | – | – | " | " | – | – | – | – | – | – | – | – |
| 2 | – | – | " | " | – | – | – | – | – | – | – | – |
| 3 | – | – | " | " | – | – | – | – | – | – | – | – |
| 4 | – | – | " | " | – | – | – | – | – | – | – | – |
| 5 | – | – | " | " | – | – | – | – | – | – | – | – |
| 6 | – | – | " | " | – | – | – | – | – | – | – | – |
| 7 | – | – | " | " | – | – | | | – | – | | |
| 8 | – | – | " | " | | | | | | | | |
| 9 | | | " | " | | | | | | | | |
| Whole serum | 100 | 190 | 190 | 380 | 100 | 190 | 100 | 190 | 50 | 190 | 100 | 190 |
| Peak I | 50 | 140 | NT | NT | 30 | 70 | 40 | 120 | 40 | 90 | 20 | 50 |
| Peak II | 120 | 110 | " | " | 190 | 150 | 150 | 130 | 50 | 80 | 160 | 220 |
| Total | 170 | 250 | " | " | 220 | 220 | 190 | 250 | 90 | 170 | 180 | 270 |
| % I | 29% | 56% | " | " | 14% | 32% | 21% | 48% | 44% | 53% | 11% | 19% |
| % II | 71% | 44% | " | " | 86% | 68% | 79% | 52% | 56% | 47% | 89% | 81% |
| I : II | 0.4 | 1.3 | " | " | 0.2 | 0.5 | 0.3 | 0.9 | 0.8 | 1.1 | 0.1 | 0.2 |

B  = *balcanica*

H  = *hardjo*

expressed as a mean percentage distribution in Figure 9.2. More than 50% of *balcanica* agglutinating activity was attributable to IgG by day 120 p.i. This level was not reached by *hardjo* IgG agglutinins until day 360 p.i. There was a rapid transition of the majority of *balcanica* agglutinating activity from the IgM to the IgG antibody class between days 60 and 120 p.i. The distribution stabilised after this time and, from day 120 to day 360 p.i., the percentage increase in *balcanica* agglutinating activity attributable to IgG relative to IgM antibody was 20%.

The mean distribution of *balcanica* and *hardjo* agglutinins in elution profiles of fractionated sera from experimentally-infected possums (Table 9.12. and Figure 9.3.) demonstrates the persistent paradoxical agglutinating activity of *hardjo* antibodies of the IgM class. This reaction was not present in *hardjo* antibodies of the IgG class, even when relatively high levels of IgG were present in fractionated sera from possums that had been infected for more than six months.

Changes in the mean agglutinating activity of IgM and IgG antibodies with increasing time after inoculation are shown in Figure 9.4. Peak IgM levels were recorded on day 15 p.i. and then fell rapidly. The relative decrease in agglutinating activity of IgM antibodies after day 120 p.i. was much less than that immediately following the attainment of peak levels. The agglutinating activity of IgG antibodies increased gradually after day 60 p.i. and then stabilised. The mean activity of *balcanica* IgG antibodies was higher than the heterologous activity of *hardjo* IgG antibodies. There was little change after peak levels were reached.

Fractionation of *balcanica* antisera produced in rabbits did not reveal a paradoxical reaction to *hardjo* in the agglutinating activity of IgM antibodies (Table 9.13.). The agglutinating activity of *balcanica* and *hardjo* antibodies of both the IgM and IgG class was higher against homologous than heterologous antigen. A similar situation was observed in whole serum from rabbits inoculated with *balcanica* and *hardjo*.

Figure 9.2. : Mean percentage distribution and standard errors of *balcanica* and *hardjo* agglutinating antibodies of the IgM and IgG class in experimentally infected possums.
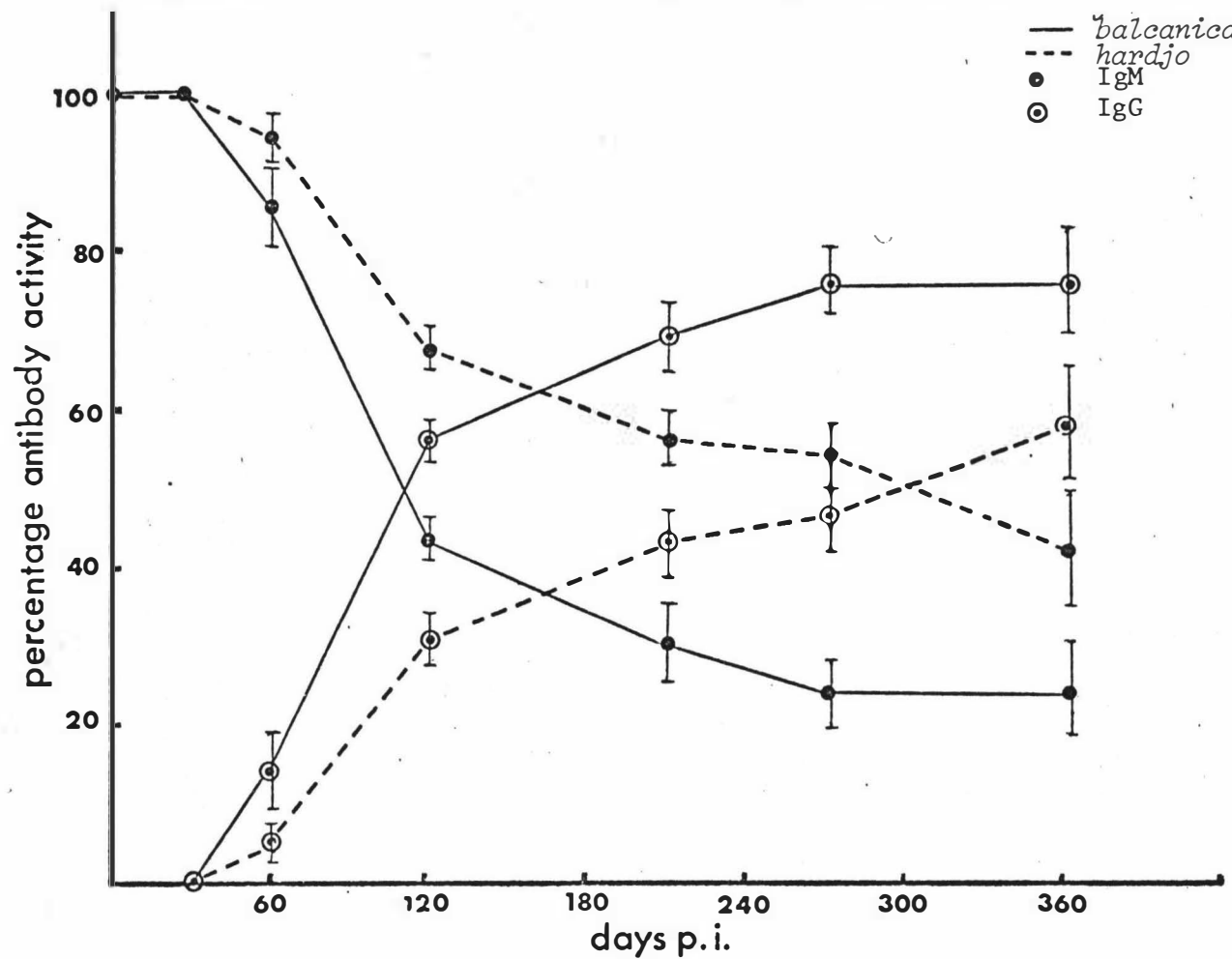
Table 9.12. : Mean distribution of *balcanica* and *hardjo* agglutinins in fractionated sera from experimentally-infected possums.

Days post-inoculation

| Fraction Number | day 15 p.i. | | day 28 p.i. | | day 60 p.i. | | day 120 p.i. | | day 210 p.i. | | day 270 p.i. | | day 360 p.i. | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | B | H | B | H | B | H | B | H | B | H | B | H | B | H |
| I 1 | 10 | 30 | 10 | 30 | – | 20 | – | 10 | – | 10 | 10 | 10 | – | – |
| 2 | 50 | 190 | 50 | 130 | 10 | 70 | 20 | 30 | 20 | 50 | 10 | 30 | 10 | 30 |
| 3 | 270 | 380 | 70 | 380 | 50 | 190 | 30 | 100 | 20 | 70 | 20 | 50 | 10 | 30 |
| 4 | 190 | 380 | 20 | 130 | 30 | 100 | 20 | 50 | 10 | 30 | 10 | 20 | 10 | 20 |
| 5 | 50 | 190 | 10 | 70 | 10 | 50 | 10 | 30 | – | 10 | – | 10 | – | 10 |
| 6 | 10 | 70 | – | 20 | – | 30 | – | 10 | – | – | | | – | – |
| II 1 | – | 10 | – | – | – | – | – | – | – | 10 | 20 | 10 | 10 | 10 |
| 2 | – | – | – | – | 10 | 10 | 10 | 20 | 20 | 20 | 30 | 30 | 30 | 30 |
| 3 | – | – | – | – | 10 | 10 | 20 | 20 | 30 | 30 | 50 | 30 | 30 | 30 |
| 4 | – | – | – | – | – | – | 20 | 30 | 50 | 50 | 30 | 20 | 20 | 30 |
| 5 | – | – | – | – | – | – | 20 | 20 | 20 | 20 | 10 | 10 | 20 | 20 |
| 6 | – | – | – | – | – | – | 10 | 10 | 20 | 10 | 10 | – | 10 | – |
| 7 | – | – | – | – | – | – | 10 | – | 10 | – | 10 | 10 | – | – |
| 8 | – | – | – | – | – | – | 10 | – | – | – | | | – | – |
| 9 | – | – | – | – | – | – | – | – | – | – | | | | |
| III 1 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 2 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 3 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 4 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 5 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 6 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 7 | – | – | – | – | – | – | – | – | – | – | – | – | – | – |
| 8 | – | – | – | – | – | – | – | – | – | – | | | – | – |
| 9 | – | – | – | – | – | – | – | – | | | | | | |
| Peak I | 580 | 1240 | 160 | 760 | 100 | 460 | 80 | 230 | 50 | 170 | 50 | 120 | 30 | 90 |
| Peak II | – | 10 | – | – | 20 | 20 | 100 | 100 | 150 | 140 | 160 | 110 | 120 | 120 |

B = *balcanica*

H = *hardjo*
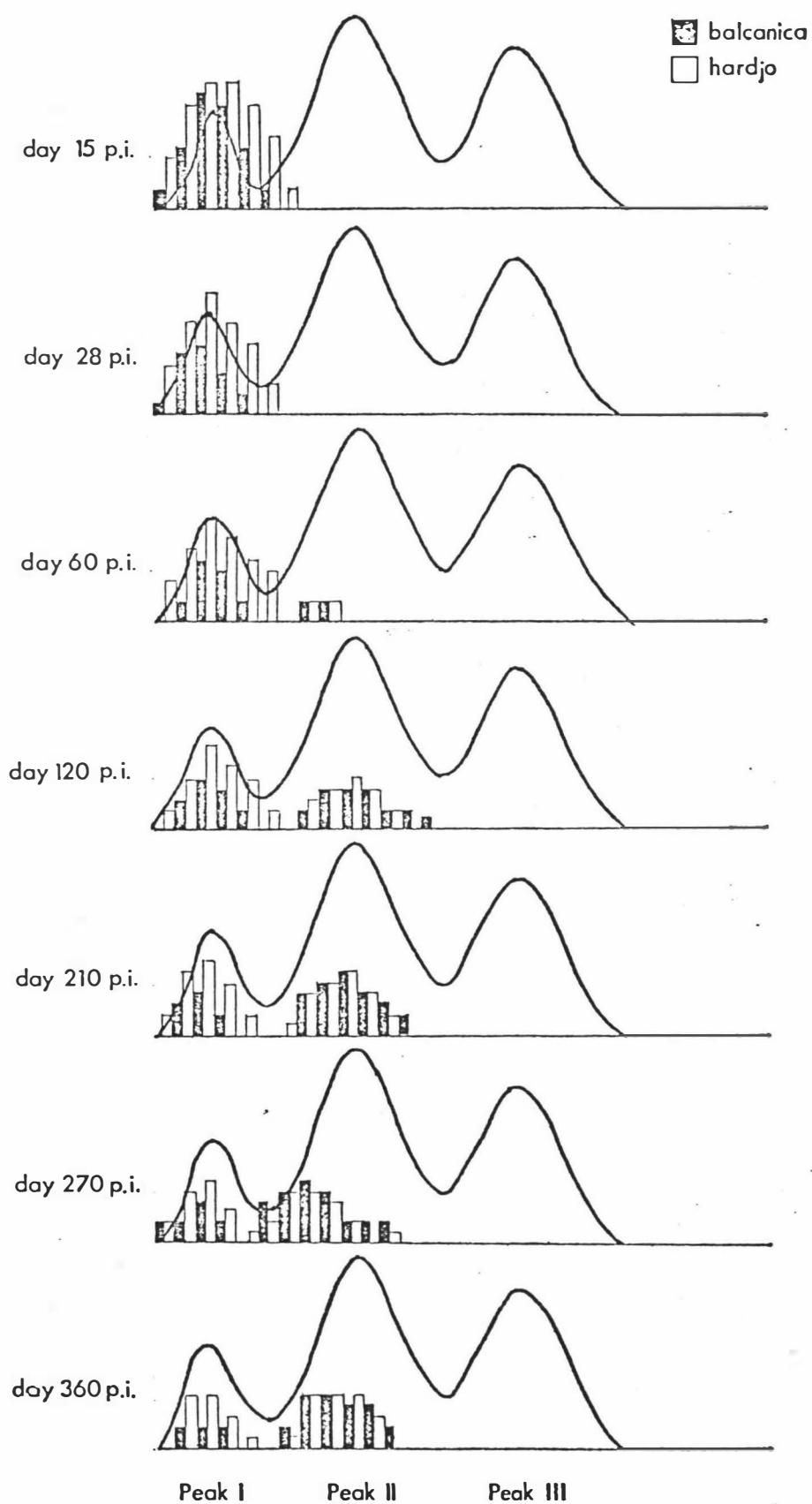
Figure 9.3. : Mean distribution of *balcanica* and *hardjo* agglutinins
in fractionated sera from experimentally infected
possums.

Figure 9.4. : Changes in mean agglutinating activity of anti-*balcanica* and anti-*hardjo* IgM and IgG immunoglobulins with time in possums experimentally infected with *balcanica*.
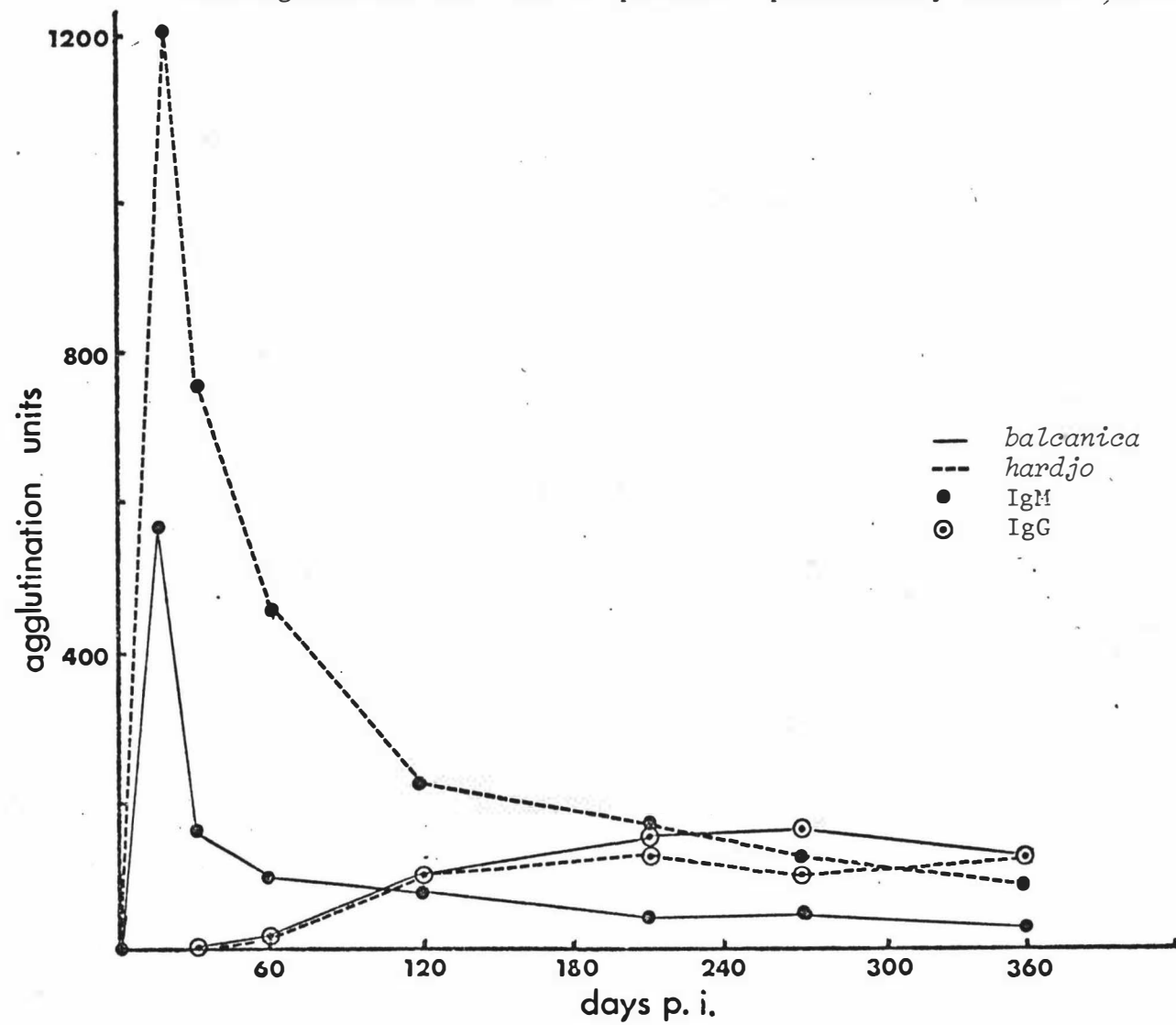
Fractionation of a random selection of sera from possums experimentally infected with *balcanica* in Experiment 2 of Chapter VII produced similar results to those described. Of special interest was the serum collected from possum E2.5 two weeks p.i. This was the only serum sample from this animal that had a *balcanica* titre, yet leptospires were recovered by kidney culture 10 weeks after inoculation. This short-lived serological response was found to be due solely to IgM antibodies.


DISCUSSION

The present study showed that antibodies present in the sera of possums naturally infected with *balcanica* were of both the IgM and IgG classes. Thus the serological response to infection in possums involves similar immunoglobulins to those described for eutherian mammals and chickens (Uhr, 1964). It has been reported that antibodies of the IgM class predominate in the agglutinating activity of anti-leptospiral sera (Hartmann *et al*, 1964; Hocker and Bauer, 1965), however, most workers report a transition from predominantly IgM to predominantly IgG antibodies within a few weeks of sero-conversion (Pike *et al*, 1965; Crawford, 1972; Chang and Faine, 1974; Chernukha *et al*, 1976). This transition was also seen in possums.

The MAT detected agglutinating activity in gel filtration-derived fractions containing IgM and IgG. It has been claimed by some workers that the MAT measures only IgM activity in sera from animals with leptospiral infection (Hanson *et al*, 1972; Hanson, 1976; 1977). This claim was not supported either by this investigation or by those relating to a variety of other species (Graves and Faine, 1970; Negi *et al*, 1971; Crawford, 1972; Chernukha *et al*, 1976).

Maternally-derived antibodies in sera from pouched-young and juvenile possums were of the IgG class. The transmission and persistence of passively-derived maternal antibodies has been discussed in Chapter XIII. Yadav (1971) found that milk from female quokka (another Australian marsupial) contained only IgG antibodies. It was also found that column chromatography-derived IgM administered to pouched

Table 9.13. : Distribution of *balcanica* and *hardjo* agglutinins in fractionated *balcanica* and *hardjo* antisera.

| Fraction Number | Rabbit anti-*balcanica* | | Rabbit anti-*hardjo* | |
|---|---|---|---|---|
| | B | H | B | H |
| I 1 | 130 | 20 | 50 | 130 |
| 2 | 130 | 50 | 100 | 100 |
| 3 | 190 | 100 | 100 | 270 |
| 4 | 380 | 100 | 130 | 380 |
| 5 | 380 | 70 | 100 | 130 |
| 6 | 380 | 100 | 70 | 100 |
| 7 | 270 | 70 | | |
| II 1 | 100 | 50 | 190 | 270 |
| 2 | 380 | 100 | 190 | 190 |
| 3 | 380 | 70 | 190 | 380 |
| 4 | 770 | 100 | 190 | 380 |
| 5 | 1540 | 100 | 380 | 380 |
| 6 | 1540 | 190 | 190 | 270 |
| 7 | 1540 | 190 | 100 | 190 |
| 8 | 770 | 190 | 70 | 70 |
| 9 | 380 | 100 | 50 | 30 |
| 10 | 190 | 100 | 20 | 20 |
| III 1 | 70 | 30 | 20 | – |
| 2 | 50 | 20 | – | – |
| 3 | 20 | – | – | – |
| 4 | – | – | – | – |
| 5 | 20 | 20 | – | – |
| 6 | 20 | – | – | – |
| 7 | – | – | – | – |
| 8 | – | – | – | – |
| 9 | – | – | – | – |
| 10 | – | – | – | – |
| Whole serum | 12290 | 3070 | 3070 | 6140 |

B = *balcanica*

H = *hardjo*

young quokka was not absorbed across the gut wall. This may also be the situation in possums. Passive transmission of maternal IgM to offspring in colostrum occurs in bovines (Porter, 1972; McGuire *et al*, 1976) and pigs (Locke *et al*, 1964). It does not occur in humans (Smith, 1960) or rats (Morris, 1967).

Fractionation of serial serum samples from possums experimentally infected with *balcanica* demonstrated that the initial serological response to infection involved only IgM antibodies. IgG antibodies were first detected on day 60 p.i., when 50% of possums had trace amounts of agglutinating activity in Peak II of Sephadex G200 elution profiles. By day 120 p.i., 50 to 62% of *balcanica* agglutinating activity was attributable to IgG and this increased to 56 to 89% by day 360 p.i. This transition from IgM to IgG followed the same pattern but was considerably slower than that described in other species. Uhr (1964) reported that IgG antibodies were detected within seven days of seroconversion in guinea pigs, and IgG was the predominating antibody class by day 40. Pike *et al* (1965) found at 44 days that 83% of the agglutinating activity in rabbit sera was attributable to IgG. Hellstrom (1978) infected calves with *hardjo* and by 42 days p.i., 60% of the agglutinating activity in sera was due to IgG. Similar results were reported by Crawford (1972) in rabbits. The late appearance of IgG and the slow transition from a predominantly IgM to a predominantly IgG antibody response in possum sera is therefore an obvious difference between the serological response of this marsupial and that of eutherian mammals.

It is possible that the delayed transition of IgM to IgG antibodies in possums infected with *balcanica* may have been partly due to the serovar involved. Chernukha *et al* (1976) reported that IgG antibodies were not detected in sera from humans infected with leptospires of the Hebdomadis serogroup until 30 days after infection took place. In comparison, the synthesis of IgG antibodies in humans infected with leptospires of the Grippotyphosa serogroup occurred within two to four days of the appearance of IgM antibodies.

Chernukha *et al* (1976) also found that the antibody response in some humans involved only IgM or only IgG antibodies. This variation was not found in possum sera. The class distribution and persistence of *balcanica* agglutinins were similar in all animals.

All fractionated sera and whole sera were tested against *hardjo* as well as *balcanica*. The consistent paradoxical reaction to *hardjo* that occurred in whole sera was attributable to a higher level of heterologous IgM agglutinating activity against *hardjo* than the level of homologous IgM activity against *balcanica*. The occurrence of a paradoxical *hardjo* reaction was independent of both the concentration of IgM relative to IgG and the time after seroconversion. IgG agglutinins in *balcanica*-infected possums had similar activity for *hardjo* as for *balcanica*. This similar agglutinating activity was also apparent in sera from juveniles that contained passively-derived IgG antibodies.

The occurrence of paradoxical reactions to a variety of serovars has been reported previously (Alston and Broom, 1958; Menges *et al*, 1960; Roth *et al*, 1963; Minette and Shaffer, 1968), however no explanation as to the immunological basis of these reactions has been proposed. A possibility that the paradoxical reaction to *hardjo* in possums infected with *balcanica* may in part be due to the poor immunogenicity of *balcanica* was suggested in Chapter VI. Fractionation studies demonstrated that the decrease in the strength of the paradoxical *hardjo* reaction in whole serum with increasing time after seroconversion (Figure 7.8.) was due to a decrease in the ratio of IgM:IgG. The dependance of paradoxical reactions on immunoglobulins of the IgM class was also reported by Chernukha *et al* (1976). These workers found that 7% of humans with leptospirosis had paradoxical reactions to heterologous antigens before the appearance of homologous agglutinins was detected. After the appearance of homologous agglutinins in the first 11 days of infection, 44 of 225 patients (20%) had paradoxical reactions to heterologous antigens. These inter-group paradoxical reactions were attributed exclusively to IgM antibodies. It is note-worthy that paradoxical IgM antibody reactions in possums occurred only with an antigen of the same serogroup as the infecting serovar. No titres were recorded against antigens of other serogroups.

Paradoxical reactions were not seen in rabbits artifically immunised with *balcanica* and *hardjo*, or in sheep and cattle experimentally infected with *balcanica* and *hardjo* (unpublished data). Thus this immunological phenomenon appears to be restricted to *balcanica* infection in possums only.

The consistent occurrence of paradoxical *hardjo* reactions resulted in higher IgM:IgG ratios for *hardjo* than *balcanica* agglutinins at all stages of experimental infections. The mean percentages of IgM and IgG antibody activity in sera one year after inoculation were 24% and 76% for *balcanica* and 42% and 58% for *hardjo*. The only other study that has monitored the change in IgM:IgG ratios for a year following seroconversion was carried out by Hellstrom (1978) who found that the transition of *hardjo* antibodies from IgM to IgG in bovines infected with this serovar was complete in some animals one year after seroconversion. Hellstrom (1978) also found that a number of sera from adult cows contained only IgG agglutinins. In experimental possums, significant proportions of IgM antibodies remained in sera collected one year after inoculation with *balcanica*. The slow rate of decrease in IgM agglutinating activity from day 210 p.i. to day 360 p.i. indicates that this class of antibody is synthesised for long periods in the possum. It has been suggested that a persistent IgM response is related to the continuing presence of antigen in the host (Uhr, 1964). This does not appear to be the case in possums. Only three of six animals (50%) were bacteriologically-positive at the termination of the experiment, yet all animals had persistent IgM agglutinating activity.

SUMMARY AND CONCLUSIONS

1.  *Balcanica* agglutinins in possums either naturally or experimentally infected with this serovar are of both the IgM and IgG class.

2.  Maternally-derived antibodies in sera of pouched-young and juvenile possums are of the 7S (IgG) class.

3.  The initial immune response to infection with *balcanica* involved antibodies of the IgM class only.

4. The transition of the predominant agglutinating activity in possum sera from antibodies of the IgM class to antibodies of the IgG class was slower than that described for eutherian mammals.

5. Significant proportions of agglutinating activity were still attributable to IgM antibodies one year after seroconversion. IgM may be synthesised in possums, independently of antigenic stimulation, for considerable periods.

6. A consistent paradoxical reaction to *hardjo* occurred in sera from possums infected with *balcanica*.  Fractionation of sera showed that this paradoxical agglutinating activity was due to IgM antibodies.

7. Equivalent cross-reactions between *hardjo* and *balcanica*, but no paradoxical reactions, were observed in serum fractions containing antibodies of the IgG class.

CHAPTER X

## AN *IN VITRO* TEST FOR THE DIFFERENTIATION OF SEROVARS *BALCANICA* AND *HARDJO*.

### INTRODUCTION

*Balcanica* and *hardjo*, , the only Hebdomadis serogroup leptospires known to be present in New Zealand, are antigenically similar and it has been shown in other chapters that infection with either of these serovars cannot be differentiated serologically. At present, the only means of differentiating between *balcanica* and *hardjo* isolates is by cross- agglutination absorption, a tedious and time-consuming procedure that is performed in reference laboratories. As any epidemiological investigation of Hebdomadis serogroup infections in animals and man in New Zealand requires that *balcanica* and *hardjo* isolates be differentiated, a simple *in vitro* test for this purpose would be of great benefit.

Attempts to develop an *in vitro* test were based on the biochemical activities of *balcanica* and *hardjo* isolates, as biological methods (inoculation of laboratory animals) gave inconsistent results. The possibilities of classification of the leptospires by biochemical methods are limited (Anon , 1967), although some differences have been shown between serovars (Russell, 1956; Ellinghausen and Sandvik, 1965; Stalheim, 1971; Mohn, 1976). A major problem is the inconsistent nature of the biochemical activity of different strains of the same serovar (Faine, 1960; Kmety and Bakoss, 1961; Stalheim, 1971). While initial attempts in the present study to differentiate *balcanica* and *hardjo* by means of catalase (Faine, 1960) DNAse (Liven, 1975) or tributyrinase activity (Ellinghausen and Sandvik, 1965) were unsuccessful, an investigation of the haemolytic activity of the two serovars gave promising results. A variety of serovars have been investigated for haemolytic activity (Russell, 1956; Kmety and Bakoss, 1961; Kasarov, 1970) and the most consistent results have been found with serovar *pomona*.

## MATERIALS AND METHODS

### Organisms

The *balcanica* isolates used in the haemolytic test had been recently isolated from possums and were maintained by weekly subculture in liquid EMJH. The bovine *hardjo* isolates had been recently isolated from cattle. The human *hardjo* isolate (NHI 2/309) had been maintained in liquid EMJH by serial subculturing for two years. A recent isolate of *pomona* from a pig was used as a positive control and *ballum*, a non-haemolytic serovar (Russell, 1956), was used as a negative control.

All the isolates used in the haemolytic test were serially subcultured simultaneously and periodically checked for bacterial contamination. Only cultures of similar density were used in each experiment.

### Blood Agar Plates

Blood was collected from humans (blood group 'O'), cattle and sheep by venepuncture into EDTA blood-collecting tubes[a]. Possums and hamsters were bled by cardiac puncture. A plasma sample from each donor was tested against *balanica, hardjo, pomona, ballum, tarassovi* and *copenhageni* by the MAT. Blood was stored at $4^\circ$C and was discarded if not used within three weeks of collection.

Red blood cells (RBC's) were washed three times in sterile, phosphate buffered, 0.85% saline (1 : 4 v/v) and the packed cells were then resuspended in sterile saline to give a 50% v/v suspension. Blood agar plates were made by pouring 10 mls of a 5% v/v suspension of RBC's in blood agar base [b] onto 10 mls of agar salt base. Blood agar plates using unwashed RBC's (10% v/v)

[a] Vacutainer, Becton-Dickenson and Co., U.S.A.

[b] B.B.L., Division of Becton - Dickenson and Co., U.S.A.

were prepared in a similar manner. Eight or ten wells were made in each plate using a sterile cork borer with an eight mm diameter. Plates that were not used within three weeks of preparation were discarded.

## Plate haemolytic test

Each well was loaded with 0.75 µl of culture using a graduated automatic pipette fitted with a sterile, disposable tip [c]. A media control was included in all plates. Plates were incubated at 30°C for 72 hours in sealed containers. Shorter incubation times resulted in a lesser degree of haemolysis and longer incubation times resulted in irregular zones of haemolysis.

Zones of haemolysis were measured with a Leitz measuring magnifier [d]. The measurement (to the nearest 0.1 mm) was taken from the edge of the well to the edge of the zone of complete haemolysis. A small, outer zone of diffuse haemolysis was occasionally seen but was not included in the test measurement.

## Tube haemolytic test

The six or seven day cultures in liquid EMJH media that were used for the tube haemolytic test were made isotonic by the addition of 0.42 mls of 10% NaCl to five mls of culture. One ml of isotonic culture and one ml of 5% washed human RBC's were incubated at 37°C for 18 hours in sterile tubes. The RBC's were dispensed with a Cornwall syringe and two replicates of each culture were tested. An osmotic haemolysate (one ml of 5% RBC's and one ml of sterile distilled water) and a medium control (one ml of 5% RBC's and one ml of isotonic medium) were incubated with each series of isolates tested.

[c]Gilson, Arnouville Les Gonesse, France

[d]Ernst Leitz, GMBH, Wetzlar, Germany

After incubation, all suspensions were diluted 1:1 with saline and the optical density (O.D.) was read at 540 nm. The spectro-photometer [e] was zeroed for 100% transmittance with a blank of an equal volume of sterile saline and medium

## Preparation of culture supernatants

Culture supernatants from two *balcanica* strains (T78 and MU1) and two *hardjo* strains (2/309 and 543) were tested for haemolytic activity in the plate test. One litre of each strain was grown to a density of approximately $10^8$ leptospires/ml in a 3.5 litre flask. The cultures were centrifuged at 20,000 G for 30 minutes and the supernatants harvested and precipitated with $(NH_4)_2SO_4$ (45 to 50% saturation) at $4^oC$. The precipitate was centrifuged at 4000 G for ten minutes and dissolved in 0.85% NaCl to 1/10 of the original volume. The solution was then successively dialysed against tap water, distilled water and physiological saline at $4^oC$, before being concentrated ten-fold by dialysis against polyethylene glycol (M.W. 20,000).

A similar volume of uninoculated medium was subjected to the same precipitation and dialysis procedures. The test for haemolytic activity of culture supernatants was performed in the same way as described for live cultures.

## Preparation of absorbed antisera

A total of 19 *balcanica* and 7 *hardjo* strains were tested for haemolytic activity. Cross-agglutination absorption studies at the W.H.O. Reference Laboratory, Atlanta, U.S.A., confirmed the identity of seven of the *balcanica* and two of the *hardjo* strains. The remaining strains were not subjected to this procedure.

[e] 'Spectronic 20', Bausch and Lomb, Rochester, U.S.A.

To differentiate between *hardjo* and *balcanica* strains, *balcanica* antiserum absorped with *hardjo* antigen and *hardjo* antiserum absorped with *balcanica* were prepared. Strains T78 (*balcanica*) and NHI 2/309 (*hardjo*) were used as antigens and antisera were produced in rabbits as described in Appendix I. The homologous titres of *hardjo* and *balcanica* antisera were 1:24,576 and 1:12,288 respectively.

Five hundred mls of culture was grown to a density of $10^8$ organisms/ml in liquid EMJH and then centrifuged at 18,000 G for 20 minutes. The supernatant was discarded and the packed leptospires were resuspended to 2% of the original culture volume with sterile, phosphate-buffered, physiological saline. This antigen suspension was standardised against a MacFarland No. 10 standard (Kmety *et al*, 1970). Agglutinins were absorbed by adding antigen to antiserum in the ratio of 21:1, according to the technique of Kmety *et al*, 1970. The serum-antigen suspension was then shaken and left to stand at room temperature for 90 minutes. The absorbed antiserum was obtained by centrifugation of this suspension. Absorptions were considered complete when agglutinins were removed completely by the homologous antigen.

Experiment 1:  Haemolytic activity of *balcanica* and *hardjo* isolates for human, bovine, sheep, possum and hamster RBC's

Three, seven and twelve day cultures of sixteen strains of *balcanica* and two strains of *hardjo* were tested against washed human, bovine, sheep, possum and hamster RBC's. *Pomona* and *ballum* isolates were included as positive and negative controls respectively. The experiment was conducted using three series of blood agar plates (Table 10.1). RBC's from the same donors were used in series I and II and RBC's from different donors were used in Series III. The New Zealand *balcanica* type strain, T78, was included in each series. Three, seven and twelve day cultures of a particular isolate were serially sampled from the

same culture tube. Media controls were included in all plates.

Experiment 1 was also duplicated using plates made from whole blood from the different species (Table 10.2).

Experiment 2:  Haemolytic activity of *balcanica* and *hardjo* isolates for human and bovine RBC's

The most consistent results in Experiment 1 were achieved with human and bovine RBC's and therefore the experiment was repeated using human and bovine RBC's from different donors (Table 10.3). Several additional *balcanica* strains and another *hardjo* strain were also tested in this experiment. Plates incorporating whole blood from the same donors were used in parallel to the plates made from washed RBC's (Table 10.4).

Experiment 3:  Haemolytic activity of recently isolated and serially-passaged *balcanica* and *hardjo* isolates.

Duplicate cultures of *balcanica* and *hardjo* strains were tested against washed human and bovine RBC's from different donors (Table 10.5). This experiment was repeated approximately one year later with the same *balcanica* and *hardjo* strains. During the intervening period these strains had been serially subcultured in liquid EMJH at weekly intervals.

Experiment 4:  Tube haemolytic test

The tube haemolytic test was used as an alternative method to test the haemolytic activity of *balcanica* and *hardjo* . Six *balcanica* and six *hardjo* strains were tested and one *ballum* strain was included as a negative control (Table 10.7). Three series of tests were run, each using RBC's from a different human donor. Plasma from all RBC donors were negative when tested by the MAT against *balcanica, ballum, copenhageni, pomona* and *tarassovi*. Each series of tests was conducted at a different time using different subcultures of the strains that were tested.

Experiment 5: <u>Haemolytic activity of culture supernatants</u>

Culture supernatant concentrates of two *balcanica* , two *hardjo* and one *pomona* strain were tested in the plate haemolytic test against washed and whole human and bovine RBC's as previously described. Uninoculated media concentrates were used as controls.

Experiment 6: <u>Absorped antiserum screening of isolates</u>

A total of 19 *balcanica,* 8 *hardjo,* 2 *pomona* and 1 *ballum* strains were tested against absorbed *balcanica* and *hardjo* antisera according to the protocol shown in Table 10.9. The MAT was performed as previously described for serogroup identification of leptospiral isolates (Chapter III).

<u>RESULTS</u>

The results of Experiment 1 demonstrated that *balcanica* cultures were haemolytic for washed human, bovine, sheep, possum and hamster RBC's (Table 10.1). All *balcanica* strains tested had haemolytic activity. *Pomona* , a recognised haemolytic serovar, had similar activity to the *balcanica* strains. *Hardjo* cultures had no haemolytic activity on blood agar plates. *Ballum* cultures were also negative.

The haemolytic activity of *balcanica* cultures was maximal at the peak phase of growth (day 7). Day 3 and day 12 cultures showed a variable haemolytic activity. There was no significant difference between haemolysis of RBC's from ruminant and non-ruminant species.

A media reaction against washed RBC's was seen with 17 of 39 media controls in Experiment 1. This occurred sporadically against RBC's of all species and consisted of a diffuse zone of incomplete haemolysis around wells containing media. Complete haemolysis was seen with 7 or 39 media controls (18%). Zone sizes for this media reaction were variable (range 0.2 to 2.2 mm).

Table 10.1 : Haemolysis of washed human, bovine, sheep, possum and hamster RBC's (Experiment 1)

| Test series | Serovar | Strain | 3 day culture | | | | | 7 day culture | | | | | 12 day culture | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Hu | B | Sh | P | Ha | Hu | B | Sh | P | Ha | Hu | B | Sh | P | Ha |
| | *balcanica* | T78 | 1.8** | 1.0 | 1.0 | C | 1.1 | 1.2 | 1.4 | 1.4 | 1.2 | 0.3 | 0.2 | 0.4 | 0.5 | 0.3 | 0.5 |
| | " | A58 | 1.0 | 0.4 | 0 | C | 0.9 | 1.0 | 1.1 | 1.3 | 1.1 | 0 | 0.2 | 0 | 0.2 | 0.2 | 0.5 |
| | " | WR 66 | 0.8 | 0.3 | 0 | C | 1.0 | 1.2 | 1.0 | 0.6 | 0.8 | 0* | 0.3 | 0.2 | 0.3 | 0.3 | 0.5 |
| I | " | A116 | 1.1 | 0.2 | 0.2 | C | 1.4 | 1.5 | 1.6 | 1.2 | 0.9 | 0.3 | 0* | 0.1 | 0.2 | 0.4 | 0.4 |
| | *pomona* | T76 | 0.3 | 0.5 | 0.5 | C | 0 | 1.4 | 1.6 | 1.0 | 1.0 | 0.5 | 0.4 | 0.6 | 0.4 | 0.1 | 0.4 |
| | *ballum* | 104S | 0 | 0 | 0 | C | 0* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *hardjo* | 2/309a | 0 | 0 | 0 | C | 0 | 0 | 0 | 0* | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | " | 2/309b | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | " | 543 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Medium control | | | 0 | 0* | 0.2 | C | 2.2 | 0* | 0 | 2.0 | 0* | 0 | 0 | 0 | 0* | 0* | 0* |
| | *balcanica* | T78 | 1.4 | 0.4 | 0.2 | 0.7 | 1.2 | 1.4 | 0.9 | 1.0 | 0.9 | 1.5 | 0.2 | 0.3 | 0.5 | C | 0.2 |
| | " | A200 | 0.4 | 0.6 | 0* | 0.9 | 1.0 | 0.8 | 0.8 | 0.4 | 0.8 | 1.0 | 0.4 | 0.2 | 0.1 | C | 0.3 |
| | " | A45 | 1.0 | 0.6 | 0 | 1.3 | 1.2 | 1.0 | 0.9 | 1.0 | 1.2 | 1.0 | 0.2 | 0.2 | 0.2 | C | 0.5 |
| II | " | MU3 | 0.2 | 0.4 | 0 | 1.3 | 0.8 | 0.5 | 0.5 | 0.3 | 1.0 | 0.8 | 0 | 0.1 | 0 | C | 0 |
| | " | WR68 | 0.6 | 0.8 | 0 | 0.6 | 0.9 | 0.6 | 0.8 | 0.6 | 0.7 | 0.9 | 0 | 0.2 | 0 | C | 0 |
| | " | WR43 | 1.1 | 0.5 | 0 | 0.9 | 1.1 | 0.7 | 0.6 | 0.5 | 1.0 | 1.0 | 0.1 | 0.4 | 0.1 | C | 0.1 |
| | " | A60 | 0* | 0.3 | 0 | 0.4 | 0* | 0.5 | 0 | NT | 0.1 | 0.9 | 0 | 0 | 0.1 | C | 0 |
| | *hardjo* | 2/309a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | C | 0 |
| | " | 543 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | C | 0 |
| Medium control | | | 0* | 0 | 0 | 0 | 0 | 0 | 0.5 | 0* | 0* | 1.8 | 0* | 0 | 0* | C | 0* |

Table 10.1 contd.

| Test series | Serovar | Strain | 3 day culture | | | | | 7 day culture | | | | | 12 day culture | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Hu | B | Sh | P | Ha | Hu | B | Sh | P | Ha | Hu | B | Sh | P | Ha |
| | *balcanica* | T78 | 1.8 | 0.8 | 0 | 1.0 | 0.5 | C | 0.6 | 0 | 1.1 | 2.1 | 0.2 | NT | 0 | 0.2 | NT |
| | " | M564 | 0.2 | 0.3 | 0 | 0.6 | 0.6 | C | 0.3 | 0 | 0.9 | 1.7 | 0.3 | " | 0 | 0.2 | " |
| | " | A123 | 0.9 | 0.6 | 0 | 0.7 | 1.0 | C | 0.6 | 0 | 1.2 | 2.0 | 0.4 | " | 0 | 0.3 | " |
| III | " | WR28 | 0.8 | 0.7 | 0 | 0.8 | 1.0 | C | 0.6 | 0 | 1.2 | 2.0 | 0.4 | " | 0 | 0.3 | " |
| | " | W88 | 1.0 | 0.8 | 0 | 3.0 | 0.5 | C | 0.4 | 0 | 1.2 | 1.8 | 0.5 | " | 0 | 0.4 | " |
| | " | WR23 | 0 | 0 | 0 | 1.0 | 0.8 | C | 0.5 | 0 | 0.8 | 1.0 | 0.1 | " | 0 | 0 | " |
| | " | A93 | 1.0 | 0.9 | 0 | 0.8 | 1.1 | C | 0.8 | 0 | 1.2 | 1.9 | 0.3 | " | 0 | 0 | " |
| | *hardjo* | 2/309 | 0 | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 | " | 0 | 0 | " |
| | " | 543 | 0 | 0 | 0 | 0 | 0.1 | C | 0 | 0 | 0 | 0.3 | 0 | " | 0 | 0 | " |
| Medium control | | | 0 | 0* | 1.0 | 0* | 0 | C | 0 | 0 | 0* | 2.0 | 0* | NT | 0 | 0* | NT |

Hu = human RBC's  
B = bovine RBC's  
Sh = sheep RBC's  
P = possum RBC's  
Ha = hamster RBC's  
C = contaminated plate  
\* incomplete haemolysis  
\*\* zone of haemolysis measured in mm.

Washed sheep RBC's in Test Series III of Experiment I were not haemolysed by *balcanica* cultures.

Unwashed RBC's from the same donors from which washed cells were obtained in Experiment 1 showed a different pattern of haemolysis (Table 10.2). *Balcanica* cultures were haemolytic for unwashed human and hamster RBC's in all test series, however possum RBC's and sheep RBC's in Series III were insuspectible. Haemolysis of unwashed bovine RBC's by *balcanica* cultures was variable. The haemolytic activity of *balcanica* cultures was lower for unwashed RBC's, of all species that were haemolysed, than for washed cells. *Hardjo* cultures were non-haemolytic for unwashed RBC's of all species. No media reactions were seen against unwashed RBC's.

Experiment 2 involved different *balcanica* strains and different human and bovine RBC donors. Similar results were achieved as in Experiment 1 (Table 10.3). Haemolytic activity was maximal in seven day cultures and all *balcanica* cultures demonstrated activity. *Hardjo* cultures were non-haemolytic for washed human RBC's, however 5 of 16 twelve day *hardjo* cultures (31%) showed a small degree of haemolytic activity against washed bovine RBC's (0.2 to 0.3 mm). Incomplete haemolytic reactions were seen with 8 of 16 media controls (50%). One media control showed a complete haemolytic reaction (6%).

Unwashed bovine RBC's in Experiment 2 were almost completely resistant to haemolysis by *balcanica* cultures (Table 10.4). This was not the case for unwashed human RBC's. *Balcanica* cultures had similar haemolytic activity for these as washed human RBC's. *Hardjo* cultures had no haemolytic activity. Media controls were also non-haemolytic for unwashed human and bovine RBC's.

The results of Experiment 3 using human and bovine RBC's (Table 10.5) were similar to those results described for washed RBC's in Experiments 1 and 2. Small haemolytic reactions (0.2 to 0.3 mm) occurred with three day and seven day *hardjo* cultures and bovine RBC's

Table 10.2 :  Haemolysis of unwashed human, bovine, sheep, possum and hamster RBC's (Experiment 1)

| Test series | Serovar | Strain | 3 day culture | | | | | 7 day culture | | | | | 12 day culture | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Hu | B | Sh | P | Ha | Hu | B | Sh | P | Ha | Hu | B | P | Sh | Ha |
| I | *balcanica* | T78 | 1.6** | 0.3 | 0* | 0 | C | 1.0 | 0.7 | 0.3 | 0 | 0.7 | 0 | 0 | 0 | 0 | 0 |
| | " | A58 | 0.7 | C | 0 | 0 | C | 0.8 | 0 | 0 | 0 | 0.5 | 0 | 0 | 0 | 0 | 0 |
| | " | WR66 | 0.6 | 0* | 0 | 0 | C | 0.9 | 0 | 0* | 0 | 0.4 | 0.1 | 0 | 0 | 0 | 0 |
| | " | A116 | 0.6 | 0* | 0 | 0 | C | 1.4 | 0 | 0 | 0 | 1.2 | 0.1 | 0 | 0 | 0.1 | 0.1 |
| | *pomona* | T76 | 0.2 | 0.3 | 0 | 0 | C | 0.2 | 1.2 | 0.5 | 0 | 0.1 | 0.1 | 0.3 | 0 | 0 | 0 |
| | *ballum* | 1045 | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0· | 0 | 0 | 0 | 0 | 0 | 0 |
| | *hardjo* | 2/309a | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | " | 2/309b | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0. | 0 | 0 | 0 | 0 | 0 | 0 |
| | " | 543 | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | Medium control | | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| II | *balcanica* | T78 | 1.1 | 0* | 0.2 | 0 | 1.2 | 0.9 | 0.3 | 0.8 | 0 | C | 0 | 0 | 0 | 0 | 0 |
| | " | A200 | 0.5 | 0* | 0.1 | 0 | 0.7 | 1.0 | 0 | 0.9 | 0 | C | 0.2 | 0 | 0 | 0 | 0 |
| | " | A45 | 0.9 | 0* | 0.1 | 0 | 1.2 | 0.7 | 0. | 0.7 | 0 | C | 0.2 | 0 | 0· | 0 | 0 |
| | " | MU3 | 0.1 | 0 | 0 | 0 | 0.5 | 0.1 | 0 | 0.2 | 0 | C | 0 | 0 | 0 | 0 | 0 |
| | " | WR68 | 0.4 | 0* | 0 | 0 | 0.4 | 0.1 | 0 | 0.2 | 0 | C | 0 | 0 | 0 | 0 | 0 |
| | " | WR43 | 0.8 | 0* | 0 | 0 | 0.6 | 0.2 | 0. | 0.5 | 0 | C | 0.1 | 0 | 0 | 0 | 0 |
| | " | A60 | 0* | 0 | 0 | 0 | 0* | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 |
| | *hardjo* | 2/309a | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 |
| | " | 543 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 |
| | Medium control | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | C | 0 | 0 | 0 | 0 | 0 |

Table 10.2 cont.

| Test series | Serovar | Stain | 3 day culture | | | | | 7 day culture | | | | | 12 day culture | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Hu | B | Sh | P | Ha | Hu | B | Sh | P | Ha | Hu | B | Sh | P | Ha |
| III | *balcanica* | T78 | 1.0 | 0.6 | 0.1 | 0 | 0.9 | NT | 0 | 0 | 0 | 0.7 | 0 | 0 | 0 | 0 | 0 |
| | | M564 | 0 | 0* | 0 | 0 | 0 | NT | 0 | 0 | 0 | 0.6 | 0 | 0 | 0 | 0 | 0 |
| | | A123 | 0.5 | 0* | 0 | 0 | 0 | NT | 0 | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 | 0 |
| | | WR28 | 0.5 | 0* | 0 | 0 | 0.2 | NT | 0 | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 | 0 |
| | | W88 | 0.7 | 0* | 0 | 0 | 0 | NT | 0 | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 | 0 |
| | | WR23 | 0 | 0 | 0 | 0 | 0 | NT | 0 | 0 | 0 | 0.4 | 0 | 0 | 0 | 0 | 0 |
| | | A93 | 0.8 | 0 | 0 | 0 | 0.4 | NT | 0 | 0 | 0 | 0.7 | 0 | 0 | 0 | 0 | 0 |
| | *hardjo* | 2/309 | 0 | 0 | 0 | 0 | 0 | NT | 0 | 0 | 0 | | 0 | 0 | 0 | 0 | 0 |
| | | 543 | 0 | 0 | 0 | 0 | 0 | NT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Medium control | | | 0 | 0 | 0 | 0 | 0 | NT | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

Hu  = human RBC's
B   = bovine RBC's
Sh  = sheep RBC's
P   = possum RBC's
Ha  = hamster RBC's
C   = contaminated plate

*   Incomplete haemolysis
**  Zone of haemolysis(mm)

Table 10.3: Haemolysis of washed human and bovine red blood cells (Experiment 2)

| Test series | Serovar | Stain | 3 day culture | | 7 day culture | | 12 day culture | |
|---|---|---|---|---|---|---|---|---|
| | | | Hu | B | Hu | B | Hu | B |
| | *balcanica* | T78 | 1.0** | 1.4 | 1.2 | 2.0 | 1.2 | 1.0 |
| I | " | MU1 | 0.8 | 1.1 | 1.0 | 1.3 | 0.9 | 0.7 |
| | " | A116 | 0.7 | 0.6 | 1.0 | 1.2 | 0.7 | 0.9 |
| | " | A58 | 0.8 | 1.0 | 1.1 | 1.4 | 0.8 | 1.2 |
| | " | WR66 | 0.7 | 0.9 | 0.9 | 1.2 | 0.9 | 0.8 |
| | *hardjo* | 543 | 0 | 0 | 0 | 0 | 0 | 0.2 |
| | " | 551 | 0 | 0 | 0 | 0 | 0 | 0 |
| | " | 2/309a | 0 | 0 | 0 | 0 | 0 | 0·2 |
| | Medium control | | 0 | 0 | 0 | 0* | 1.0 | 0* |
| | *balcanica* | T78 | 0.9 | C | 1.3 | 2.0 | 1.4 | 1.8 |
| | " | A123 | 0.9 | C | 0.9 | 1.4 | 0.5 | 0.4 |
| | " | WR23 | 0.8 | C | 0.9 | 1.0 | 0.7 | 0.4 |
| | " | WR28 | 0.6 | C | 0.9 | 0.9 | 0.6 | 0.6 |
| II | " | W88 | 0.9 | C | 1.2 | 1.7 | 0.9 | 1.0 |
| | " | A93 | 0.8 | C | 1.0 | 1.5 | 1.0 | 1.1 |
| | *hardjo* | 543 | 0 | C | 0 | 0 | 0 | 0.3 |
| | " | 551 | 0 | C | 0 | 0 | 0 | 0 |
| | " | 2/309a | 0 | C | 0 | 0 | 0 | 0.2 |
| | Medium control | | 0* | C | 0* | 0 | 0 | 0* |
| | *balcanica* | T78 | 1.0 | C | 1.4 | 1.7 | 1.2 | 2.0 |
| | " | WR68 | 0.7 | C | 0.7 | 1.5 | 0.3 | 0.3 |
| | " | A200 | 0.6 | C | 1.2 | 1.2 | 0.6 | 0.5 |
| III | " | A45 | 0.5 | C | 0.8 | 15. | 0.7 | 0.8 |
| | " | MU3 | 0.4 | C | 0.3 | 0.3 | 0.5 | 0.6 |
| | " | WR43 | 0.6 | C | 0.7 | 0.7 | 0.6 | 0.6 |
| | *hardjo* | 543 | 0 | C | 0 | 0.1 | 0 | 0 |
| | " | 551 | 0 | C | 0 | 0 | NT | NT |
| | " | 2/309 a | 0 | C | 0 | 0 | 0 | 0.3 |
| | Medium control | | 0 | C | 0 | 0* | 0 | 0* |

Hu = human RBC's
B = bovine RBC's
C = contaminated plate
** zone of haemolysis (mm)
* zone of incomplete haemolysis (mm) .

Table 10.4: Haemolysis of unwashed human and bovine RBC's (Experiment 2)

| Test series | Serovar | Stain | 3 day culture | | 7 day culture | | 12 day culture | |
|---|---|---|---|---|---|---|---|---|
| | | | Hu | B | Hu | B | Hu | B |
| | balcanica | T78 | 0.9* | 0 | 1.2 | 0.3 | 1.0 | 0 |
| | " | MU1 | 0.8 | 0 | 0.9 | 0 | 0.6 | 0 |
| I | " | A116 | 0.4 | 0 | 0.7 | 0 | 0.4 | 0 |
| | " | A58 | 0.7 | 0 | 1.0 | 0 | 0.6 | 0 |
| | " | WR66 | 0.5 | 0 | 1.1 | 0 | 0.6 | 0 |
| | hardjo | 543 | 0 | 0 | 0 | 0 | 0 | 0 |
| | —" | 551 | 0 | 0 | 0 | 0 | 0 | 0 |
| | " | 2/309 a | 0 | 0 | 0 | 0 | 0 | 0 |
| Medium control | | | 0 | 0 | 0 | 0 | 0 | 0 |
| | balcanica | T78 | NT | 0.1 | C | 0.2 | 1.2 | 0 |
| | " | A123 | NT | 0 | C | 0 | 0.7 | 0 |
| | " | WR23 | NT | 0 | C | 0.1 | 0.8 | 0 |
| | " | WR28 | NT | 0 | C | 0 | 0.7 | 0 |
| II | " | W88 | NT | 0 | C | 0 | 0.7 | 0 |
| | " | A93 | NT | 0 | C | 0 | 0.8 | 0 |
| | hardjo | 543 | NT | 0 | C | 0 | 0 | 0 |
| | " | 551 | NT | 0 | C | 0 | 0 | 0 |
| | " | 2/309 a | NT | 0 | C | 0 | 0 | 0 |
| Medium control | | | NT | 0 | C | 0 | 0 | 0 |
| | balcanica | T78 | NT | 0.2 | C | 0.1 | 1.2 | 0 |
| | " | WR68 | NT | 0 | C | 0 | 0.3 | 0 |
| | " | A200 | NT | 0 | C | 0 | 0.1 | 0 |
| | " | A45 | NT | 0.2 | C | 0 | 0.4 | 0 |
| III | " | MU3 | NT | 0 | C | 0 | 0 | 0 |
| | " | WR43 | NT | 0 | C | 0 | 0.3 | 0 |
| | hardjo | 543 | NT | 0 | C | 0 | 0 | 0 |
| | | 551 | NT | 0 | C | 0 | 0 | 0 |
| | | 2/309 a | NT | 0 | C | 0 | 0 | 0 |
| Medium control | | | NT | 0 | C | 0 | 0 | 0 |

Hu = human RBC's
B = bovine RBC's
C = contaminated plate
* zone of haemolysis (mm)

Table 10.5:   Haemolysis of washed human and bovine red blood cells.
              (Experiment 3)

| Serovar | Strain | 3 day culture | | 7 day culture | | 14 day culture | |
| --- | --- | --- | --- | --- | --- | --- | --- |
| | | Hu | B | Hu | B | Hu | B |
| *balcanica* | A45 | 1.3** | 1.4 | 1.8 | 1.4 | 0.3 | 0 |
| " | A45 | 1.5 | 1.5 | 1.7 | 1.4 | 0.3 | 0.1 |
| " | WR23 | 1.3 | 1.1 | 1.6 | 1.1 | 0.1 | 0 |
| " | WR23 | 1.3 | 1.0 | 1.3 | 1.0 | 0.2 | 0 |
| " | WR68 | 1.3 | 1.0 | 1.3 | 1.0 | 0 | 0 |
| " | WR68 | 1.2 | 1.0 | 1.5 | 1.2 | 0.2 | 0 |
| *hardjo* | 2/309a | 0 | 0.3 | 0 | 0.2 | 0 | 0 |
| " | " | 0 | 0.2 | 0 | 0.3 | 0 | 0 |
| Medium control | | 0 | 0* | 0 | 0. | 0.5 | 0.9 |
| " | " | 0 | 0* | 0 | 0 | 0* | 0* |

Hu   = human RBC's
B    = bovine RBC's
**     zone of haemolysis (mm)
*      incomplete haemolysis (mm)

Quantitative aspects of the haemolytic activity of *balcanica* compared with *hardjo* cultures, for human and bovine RBC's in Experiments 1, 2 and 3, are presented in Tables 10.6 and 10.7 and Figures 10.1 and 10.2. There was little difference in the haemolytic activity of seven day *balcanica* cultures for washed human and bovine RBC's in the same experimental series (Table 10.6 and Figure 10.1).

The haemolytic activity of seven day *balcanica* cultures for washed RBC's from different donors was also similar. There was no significant difference between the activity for washed human RBC's from different donors in Experiments 1 and 2 (t = 0.01, P > 0.25). Similarly, there was no significant difference between the activity for unwashed human RBC's from different donors in Experiment 1 and 2. (t = 1.30, p > 0.10). Washed RBC's from the human donor in Experiment 3 were haemolysed to a greater degree than other human RBC's (Table 10.6). This increase in haemolytic activity was significantly different from that seen for washed human RBC's in Experiment 1 (t = 3.71, P < 0.001) and Experiment 2 (t = 3.58, P < 0.001).

Washed and unwashed RBC's from the same human donor showed no significant difference in haemolysis by *balcanica* cultures (t = 1.66, P > 0.05 for series I and II in Experiment 1; t = 0.26, P > 0.25 for Experiment 2). Comparative data was not available for Series III of Experiment 1 due to a contaminated, unwashed RBC plate. Unwashed bovine RBC's in Experiment 1 and 2 were almost completely resistant to haemolysis by seven day *balcanica* cultures (Table 10.2 and Figure 10.2).

The most consistent results were therefore obtained with human RBC's. Both washed and unwashed human RBC's were haemolysed to a similar degree with seven day *balcanica* cultures, whereas no haemolytic activity was seen with *hardjo* cultures.

Repeat testing against washed human RBC's of *balcanica* cultures that had been serially subcultured for longer than a year showed that there was a decrease in haemolytic activity (Table 10.8).

Table 10.6    Haemolysis of washed human and bovine red blood cells by *balcanica*, *pomona*, *hardjo* and *ballum*

| Expt. No. | Serovar | 3 day cultures | | 7 day cultures | | 12 day cultures | |
|---|---|---|---|---|---|---|---|
| | | Human RBC's | Bovine RBC's | Human RBC's | Bovine RBC's | Human RBC's | Bovine RBC's |
| 1 | *balcanica* | 0.3 ± 0.55* | 0.53 ± 0.27 | 0.95 ± 0.35 | 0.79 ± 0.33 | 0.22 ± 0.15 | 0.19 ± 0.14 |
| | *pomona* | 0.3 | 0.5 | 1.4 | 1.6 | 0.4 | 0.6 |
| | *hardjo* | 0 | 0 | 0 | 0 | 0 | 0 |
| | *ballum* | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | *balcanica* | 0.73 ± 0.17 | 1.0 ± 0.29 | 0.94 ± 0.26 | 1.3 ± 0.43 | 0.79 ± 0.29 | 0.86 ± 0.47 |
| | *hardjo* | 0 | 0 | 0 | 0.01 ± 0.03 | 0 | 0.15 ± 0.13 |
| 3 | *balcanica* | 1.32 ± 0.10 | 1.17 ± 0.22 | 1.53 ± 0.21 | 1.18 ± 0.18 | 0.33 ± 0.34 | 0.02 ± 0.04 |
| | *hardjo* | 0 | 0.25 ± 0.07 | 0 | 0.25 ± 0.07 | 0 | 0 |

* mean zone size and standard deviation (mm)

Table 10.7:    Haemolysis of unwashed human and bovine red blood cells by *balcanica, pomona, hardjo* and *ballum*

| Exp. No. | Serovar | 3 day cultures | | 7 day cultures | | 12 day cultures | |
|---|---|---|---|---|---|---|---|
| | | Human RBC's | Bovine RBC's | Human RBC's | Bovine RBC's | Human RBC's | Bovine RBC's |
| 1 | *balcanica* | 0.6 ± 0.42* | 0.05 ± 0.15 | 0.65 ± 0.47 | 0.17 ± 0.49 | 0.04 ± 0.07 | 0 |
| | *pomona* | 0.2 | 0.3 | 0.2 | 1.2 | 0.1 | 0.3 |
| | *hardjo* | 0 | 0 | 0 | 0 | 0 | 0 |
| | *balcanica* | 0 | 0 | 0 | 0 | 0 | 0 |
| 2 | *balcanica* | 0.66 ± 0.21 | 0.03 ± 0.07 | 0.98 ± 0.19 | 0.04 ± 0.09 | 0.61 ± 0.34 | 0 |
| | *hardjo* | 0 | 0 | 0 | 0 | 0 | 0 |

Figure 10.1.: Haemolytic activity of three, six and twelve day *balcanica* cultures for washed human and bovine red blood cells.

human red blood cells

---- bovine red blood cells

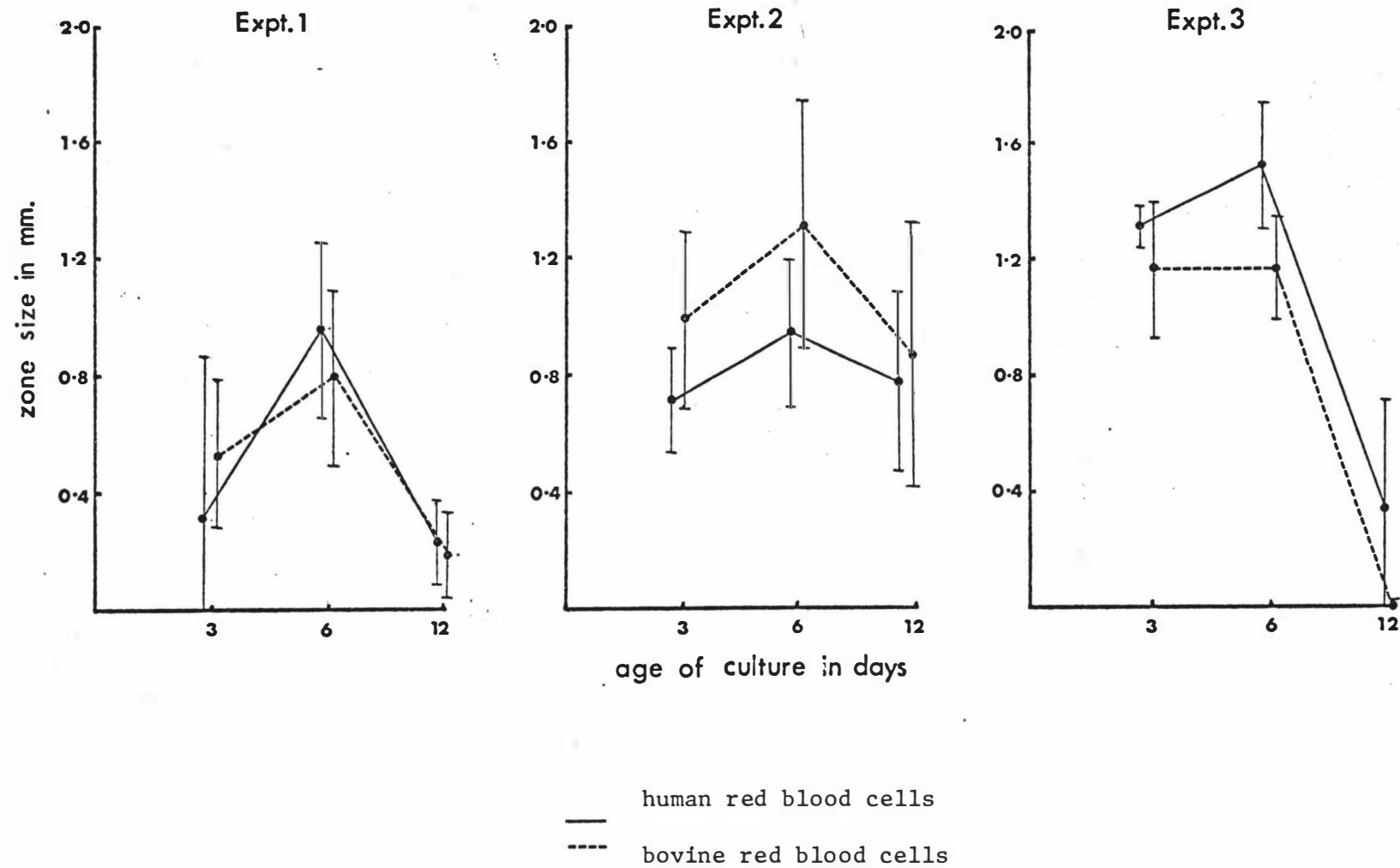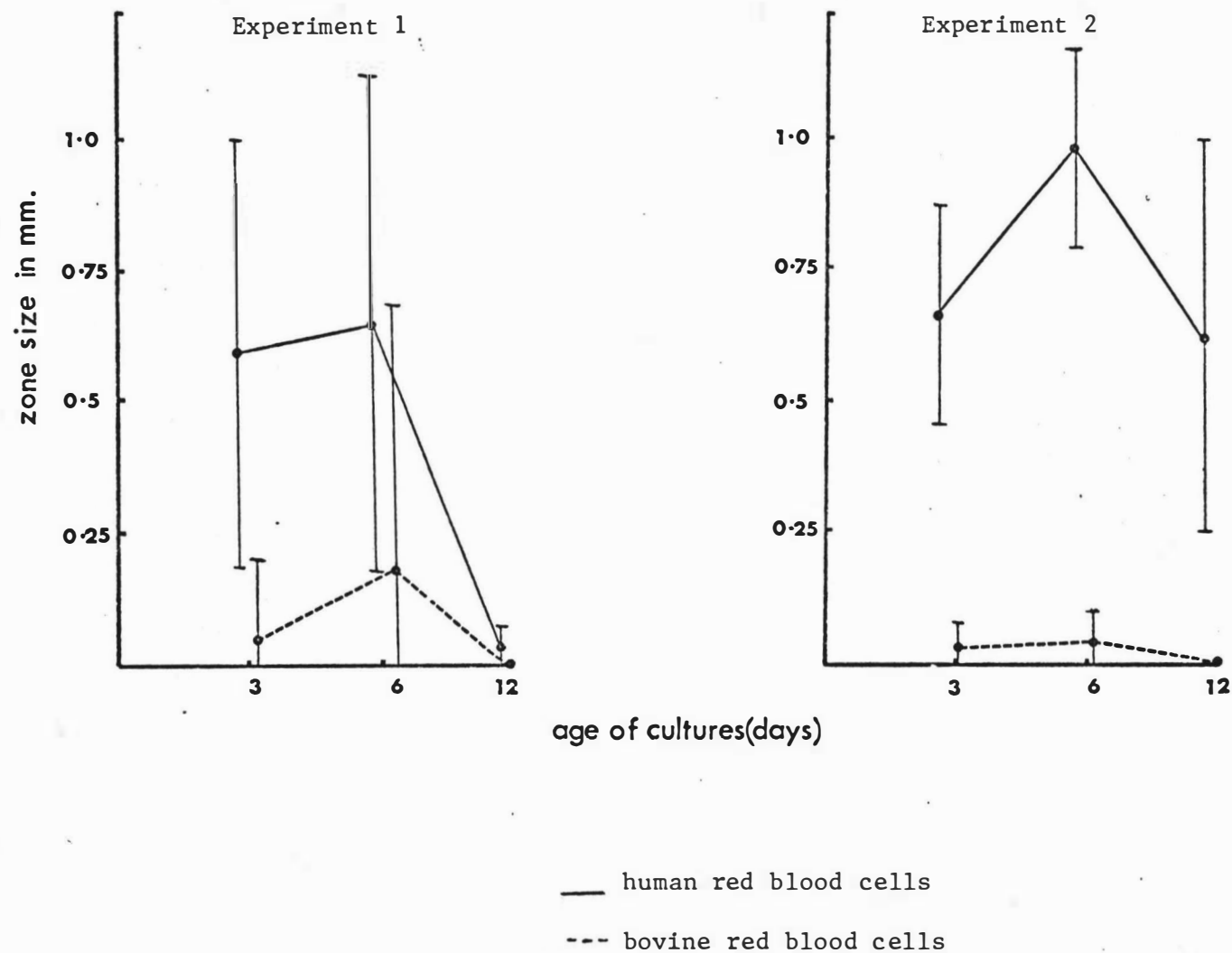Figure 10.2.:   Haemolytic activity of three, six and twelve day *balcanica* cultures
for unwashed human and bovine red blood cells.

___ human red blood cells

--- bovine red blood cells

Although activity was retained by 5 of 6 strains (83%), two of these had a diminished haemolytic effect. One strain had lost its haemolytic activity after serial subculture.

Table 10.8 Repeat testing of *balcanica* strains for haemolytic activity against washed human RBC's after serial subculturing for one year.

| Serovar | Strain | Mean haemolytic activity (mm) | |
|---------|--------|--------|--------|
| | | Test 1 | Test 2 |
| *balcanica* | T78 | 1.3 | 1.4 |
| | A45 | 1.8 | 1.0 |
| | A93 | 1.0 | 0.3 |
| | WR23 | 1.4 | 0.9 |
| | WR66 | 0.8 | 0 |
| | WR68 | 1.2 | 0.4 |
| *hardjo* | 2/309 a | 0 | 0 |
| | 543 | 0 | 0 |

Considerable variability was observed in the ability of *balcanica* cultures to haemolyse washed compared with unwashed bovine, ovine and possum RBC's. Unwashed RBC's from these species were particularly resistant to haemolysis. There was a close association between this resistance to the haemolytic effect of *balcanica* cultures and the Hebdomadis serogroup titres of the RBC donors (Table 10.9). All unwashed RBC's from donors with Hebdomadis serogroup titres were resistant to haemolysis. A partial resistance to haemolysis was apparent in unwashed bovine and ovine RBC's in Series I and II of Experiment 1 and also in unwashed bovine RBC's in Series III of Experiment 1. Hebdomadis serogroup titres were not detected in sera from these latter donors.

Table 10.9    Serum titres of RBC donors and haemolysis of RBC's by *balcanica* cultures

| Experiment | Series | Donor species | Serum titre of donor | | Haemolysis by *balcanica* | |
| --- | --- | --- | --- | --- | --- | --- |
| | | | *balcanica* | *hardjo* | washed RBC's | unwashed RBC's |
| 1 | I and II | human | 0 | 0 | + | + |
| | | bovine * | 0 | 0 | + | + − |
| | | ovine | 0 | 0 | + | + − |
| | | possum | 1:24 | 1:48 | + | − |
| | | hamster | 0 | 0 | + | + |
| | III | human | 0 | 0 | + | + |
| | | bovine | 0 | 0 | + | + − |
| | | ovine | 1:48 | 1:384 | − | − |
| | | possum | 1:96 | 1:384 | + | − |
| | | hamster | 0 | 0 | + | + |
| 2 | I, II and III | human | 0 | 0 | + | + |
| | | bovine | 1:384 | 1:768 | + | − |
| 3 | I | human | 0 | 0 | + | NT |
| | | bovine | 0 | 1:48 | + | NT |

*this animal had a *pomona* titre of 1:192

The resistance to the haemolytic effect of *balcanica* cultures was removed by washing RBC's of the respective donors.  The only exceptions to this were washed ovine RBC's in Series III of Experiment I.    These RBC's remained resistant to haemolysis after washing.

Contamination occurred in 13 of 130 blood agar plates (10%).  This was attributed to the multiple inoculation procedures for each plate that were necessary to evaluate the haemolytic test.

Concentrated *balcanica* culture supernatants showed little haemolytic  activity for human or bovine RBC's respectively.  A mean haemolytic zone size of 1.2 ± 0.26 mm for bovine RBC's and 0.8 ± 0.32 mm for human RBC's was produced with concentrated *pomona* culture supernatant.  *Hardjo* culture supernatants were negative.

The tube haemolytic test proved to be a simple and effective means of testing the haemolytic activity of *balcanica* and *hardjo* cultures.  The results using washed human RBC's from three different donors are given in Table 10.10.  The mean O.D. and standard deviation for *balcanica* cultures incubated with human RBC's was 0.61 ± 0.07.  The mean and standard deviation for *hardjo* cultures was 0.12 ± 0.04.  This was slightly lower than the mean O.D. for media controls (0.17 ± 0.02).  *Ballum* cultures had a mean O.D. of 0.09 ± 0.04.  Three 100% haemolysates had mean O.D. values of 0.87 ± 0.02.  Reference to a standard curve using mean O.D.'s for *balcanica* cultures, *hardjo* cultures and media controls gave percentage haemolysis values for human RBC's of 70%, 14% and 19% respectively.  Thus media controls gave higher percentage haemolysis values than *hardjo* cultures.

Titration of *balcanica* and *hardjo* strains against absorbed antisera confirmed the differentiation established by haemolytic testing (Table 10.11).  The titres of *balcanica* and *hardjo* strains that had not been identified by cross-agglutination absorption were within the same range as titres of *balcanica* and *hardjo* strains whose identityhad been established at the W.H.O. Reference Laboratory, Atlanta, U.S.A.

Table 10.10: Haemolysis of washed human RBC's incubated with leptospires of the Hebdomadis serogroup in a tube haemolytic test.

| Serovar | Strain | Series I Optical Density | | Series II Optical Density | | Series III Optical Density | | Mean O.D. | S.D. | S.E. |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Tube 1 | Tube 2 | Tube 1 | Tube 2 | Tube 1 | Tube 2 | | | |
| *balcanica* | T78 | 0.8 | 0.8 | 0.72 | 0.70 | 0.65 | 0.63 | 0.72 | 0.07 | 0.03 |
| | A45 | 0.75 | 0.75 | 0.75 | 0.68 | 0.49 | 0.52 | 0.66 | 0.12 | 0.05 |
| | E22 | 0.69 | 0.70 | 0.48 | 0.49 | 0.64 | 0.64 | 0.61 | 0.10 | 0.04 |
| | WR23 | 0.52 | 0.55 | 0.75 | 0.75 | 0.51 | 0.51 | 0.60 | 0.12 | 0.05 |
| | AH2 | 0.69 | 0.71 | 0.41 | 0.43 | 0.45 | 0.40 | 0.52 | 0.13 | 0.05 |
| | A93 | 0.60 | 0.62 | 0.60 | 0.50 | 0.50 | 0.52 | 0.56 | 0.06 | 0.02 |
| *hardjo* | 2/309a | 0.17 | 0.17 | NT | NT | 0.19 | 0.16 | 0.17 | 0.01 | 0.06 |
| | 2/309b | 0.06 | 0.07 | 0.09 | 0.12 | NT | NT | 0.09 | 0.03 | 0.01 |
| | RB35 | 0.05 | 0.07 | 0.06 | 0.07 | NT | NT | 0.06 | 0.01 | 0.05 |
| | RB37 | 0.11 | 0.10 | 0.08 | 0.12 | 0.18 | 0.16 | 0.13 | 0.04 | 0.02 |
| | RB55 | 0.13 | 0.10 | 0.09 | 0.10 | 0.16 | 0.17 | 0.13 | 0.04 | 0.02 |
| | 39/1 | 0.06 | 0.07 | 0.11 | 0.10 | 0.14 | 0.15 | 0.11 | 0.04 | 0.02 |
| *ballum* | BH14 | 0.04 | 0.05 | 0.07 | 0.14 | 0.10 | 0.12 | 0.09 | 0.04 | 0.02 |
| medium control | | 0.16 | 0.17 | 0.14 | 0.15 | 0.20 | 0.18 | 0.17 | 0.02 | 0.01 |
| 100% haemolysate | | 0.87 | 0.88 | 0.90 | 0.87 | 0.85 | 0.87 | 0.87 | 0.02 | 0.01 |

Table 10.11: Titration of *balcanica* and *hardjo* strains against absorbed antisera in the microscopic agglutination test.

| Serovar | Strain | reciprocal of titre against absorbed antisera | |
| | | *balc.* absorbed with *hardjo* | *hardjo* absorbed with *balc.* |
|---|---|---|---|
| *balcanica* | T78* | 1536 | 0 |
| | A45 | 1536 | 24 |
| | A58* | 768 | 24 |
| | A60* | 1536 | 24 |
| | A93* | 1536 | 24 |
| | A116 | 1536 | 48 |
| | A123 | 768 | 24 |
| | A200 | 384 | 0 |
| | AH2 | 1536 | 24 |
| | E22 | 1536 | 24 |
| | MG64 | 384 | 0 |
| | MU1* | 768 | 24 |
| | MU3 | 768 | 0 |
| | WR23* | 1536 | 48 |
| | WR28 | 1536 | 24 |
| | WR43* | 3072 | 24 |
| | WR66 | 3072 | 24 |
| | WR68 | 1536 | 24 |
| | W88 | 768 | 24 |
| *hardjo* | 2/309* | 0 | 384 |
| | 543 | 0 | 384 |
| | 551 * | 24 | 384 |
| | 39/1 | 48 | 384 |
| | RB27 | 24 | 384 |
| | RB35 | 24 | 384 |
| | RB55 | 0 | 384 |

* typed by cross-agglutination absorption at the WHO Ref. Laboratory, Atlanta, USA.

DISCUSSION

*Balcanica* cultures were haemolytic for human, cattle, sheep, possum and hamster RBC's. The haemolytic activity of leptospires demonstrated by other workers has been mainly confined to serovars of the Canicola, Grippotyphosa, Icterhaemorrhagiae and Pomona serogroups (Russell, 1956; Alexander *et al,* 1956; Kasarov, 1970). The haemolytic activity of *balcanica* has not been previously investigated although other serovars of the Hebdomadis serogroup have been shown to be non-haemolytic (Russell, 1956; Kasarov, 1970: Hodges, 1974).

All the *balcanica* isolates investigated in the present study were haemolytic, although the activity of different strains showed some variation. Other workers have reported that some strains of a recognised haemolytic serovar have had no haemolytic activity (Russell, 1956; Kmety and Bakoss, 1961; Chorvath, 1975). All strains used in the present study were recently isolated. However, it was found that the haemolytic activity of some strains that had been serially subcultured for more than a year in liquid media was either diminished or lost. As stock strains of particular serovars were often used by the above-mentioned workers to test for haemolytic activity, it is possible that long-term laboratory maintenance had resulted in some strains losing their haemolytic activity.

There was little difference between the degree of haemolysis of washed ruminant and non-ruminant RBC's by *balcanica* cultures. *Pomona* cultures were slightly more haemolytic for ruminant than non-ruminant species using the test system described in the present investigation. It is generally regarded that ruminant RBC's are more readily haemolysed by leptospires than non-ruminant RBC's (Russell, 1956; Kasarov, 1970; Kemenes 1974). In the non-ruminants, human RBC's are considered to be haemolysed more readily than rabbit or pig RBC's (Kasarov, 1970).

*Hardjo* cultures were non-haemolytic for human RBC's. A small degree of haemolysis was observed with washed bovine RBC's in Experiment

2 and 3, however, this was not constant and when it did occur it was much less than the comparative *balcanica* haemolytic reaction. The degree of haemolysis of human RBC's in the tube test was less than that of media controls. Hodges (1974) similarly found New Zealand *hardjo* isolates to be non-haemolytic.

The inability of *hardjo* to haemolyse both human and bovine RBC's formed the basis of a simple *in vitro* test to differentiate between *hardjo* and *balcanica* isolates. The most consistent results were obtained using washed human RBC's (see later discussion). While unwashed human RBC's could also be used to demonstrate the haemolytic activity of *balcanica* strains, they were less readily haemolysed than were washed RBC's. The haemolytic test could be performed using blood agar plates or as a tube test measuring optical density in a spectrophotometer. Maximum haemolytic activity was found in cultures at the peak phase of growth and therefore isolates should be well grown before tested. The precision of the test was not affected by using RBC's from different human donors.

A variable media reaction was observed against both human and bovine washed RBC's. This reaction was completely inhibited by the presence of leptospires in the culture medium. Both *hardjo* and *ballum* cultures were non-haemolytic on the same plate on which media reactions were seen. The biochemical explanation for this observation is unknown. There were no significant differences in osmolarity between media that had supported the growth of seven day *hardjo* and *ballum* cultures and unused media controls.

Several investigators have demonstrated the presence of a haemolysin in culture supernatant fluids of leptospirae (Russell, 1956; Stalheim, 1971; Chorvath, 1975). Russell (1956) found that the haemolytic activity of *pomona* culture supernatants was similar to that of whole cultures. Although leptospiral haemolysin has not been characterised, Chorvath (1975) reported that the *pomona* haemolysin in concentrated culture supernatants was similar in activity to a phospholipase. Valentine *et al* (1964) produced haemolysis of ruminant RBC's using sonicated *pomona* bacterial cells.

Concentrated *pomona* culture supernatant was haemolytic for both human and bovine RBC's in the present investigation. Concentrated *balcanica* culture supernatants had only minimal haemolytic activity. *Hardjo* supernatants were negative. The disparity between *pomona* and *balcanica* supernatants was not observed with whole cultures on blood agar plates, and the reason remains undetermined.

There was a close association between the presence of Hebdomadis serogroup titres in sera from bovine, ovine and possum RBC donors and the resistance of unwashed RBC's to haemolysis. This resistance also occurred in washed sheep RBC's in Series III of Experiment I. Unwashed human and hamster RBC's were not resistant to haemolysis by *balcanica* . These were the only two species of the RBC donors in which Hebdomadis serogroup infection was not endemic.

The ability of serum agglutinins to inhibit the haemolytic activity of leptospires has been noted by other workers. Russell (1956) and Kemenes (1974) reported that specific antisera inhibited the haemolytic activity of *pomona* cultures and supernatants. Other workers have elicited the production of haemolysin-inhibiting antibodies in experimentally-infected ruminants (Alexander, 1971; Bhasin *et al* , 1971; Hodges, 1974). It has also been demonstrated *in vitro* that haemolysin-inhibiting antibodies will cross-react with heterologous haemolysins (Alexander, 1971; Hodges, 1974). This situation was seen in the present study.

Heterologous inhibition occurred between *balcanica* cultures and unwashed RBC's from cattle and sheep infected with *hardjo* . It is interesting to speculate that this *in vitro* demonstration of heterologous protection between *balcanica* and *hardjo* agglutinins may be a reflection of a wider cross-protection that exists when animals with *hardjo* agglutinins are challenged with *balcanica.*

Absorbed antisera screening of *balcanica* and *hardjo* cultures gave a clear differentiation of these two serovars and these results fully supported the results of the haemolytic test. Although

testing of isolates against absorped antisera produced against
*balcanica* and *hardjo* was not definitive, it gave a strong indication
of serovar identity of those strains not identified at a reference
laboratory. The absorbed antisera testing of isolates as described
in this investigation does not, however, rule out the possibility
of other non-haemolytic serovars of the Hebdomadis serogroup being
present in New Zealand and being regarded as *hardjo*.

## SUMMARY AND CONCLUSIONS

1. *Balcanica* cultures were haemolytic for human, bovine, ovine,
possum and hamster RBC's. Haemolytic activity of a serovar from
the Hebdomadis serogroup has not been previously described.

2. *Hardjo* cultures were non-haemolytic for RBC's from the above-
mentioned species.

3. A simple *in vitro* blood agar plate test and spectophotometric
tube test is described for the differentiation of *balcanica* and *hardjo*
isolates. Optimal results were achieved with washed human RBC's.

4. Inconstant media reactions on blood agar plates were completely
inhibited by the presence of *hardjo* and *balcanica* cultures. The
biochemical nature of this phenomenon is unknown.

5. There was a decrease in haemolytic activity of *balcanica*
cultures that had been serially subcultured for longer than a year.

6. The haemolytic activity of *balcanica* cultures was inhibited by
unwashed RBC preparations from blood donors that had homologous or
heterologous serum titres.

7. Those isolates confirmed as either *balcanica* and *hardjo* by
cross-agglutination absorption techniques at a WHO Reference Laboratory
could also be differentiated by their haemolytic activity.

CHAPTER XI

NATURAL HISTORY OF SMALL MAMMALS IN NEW ZEALAND AND
THE METHODS OF CAPTURE USED IN THE PRESENT STUDY.

## INTRODUCTION

The long geographic isolation and special environmental cond-
itions that prevail in New Zealand have produced a unique fauna,
many of whose representatives are not found elsewhere.  The
indigenous mammalian fauna consists of only two species of bat
( *Mystacina tuberculata, Chalinolobus morio)*which are present
in small numbers and have a very localised distribution.

Since the beginning of the period of European settlement in
the late 18th Century, there has been a largely deliberate dispersal
by man of a considerable number of free-living mammalian species
from other countries.  Those exotic species which successfully
adapted to their new environment constitute the present mammalian
wildlife of New Zealand.  In many cases they have severely modified
the environment in which they live.

The most important free-living species as disseminators of
leptospirosis are the smaller mammals and it is these species which
have received the closest attention in the present study.  It is
necessary to briefly consider the natural history of the species
investigated in the present study to gain an understanding of their
importance as carriers and disseminators of leptospires in New
Zealand.

## RODENTIA

There are four species of rodents in New Zealand,with consider-
able difference in their biology and distribution.  All have occupied
vacant ecological niches to varying degrees.  The Kiore (*Rattus
exulans)*, introduced in the 14th century by Polynesians, is now
restricted to the southwest coast of the South Island and offshore
islands and therefore is of no importance in terms of the present
study.

Ship Rat (*Rattus rattus*)

The ship rat has a worldwide distribution and was inadvertently introduced to New Zealand by early seafarers. It is widely distributed throughout New Zealand in both urban and rural areas and is commonly found inhabiting the walls and roofs of houses and farm buildings (Watson, 1959; Best, 1968). In forested regions the ship rat prefers mature forest to scrub and bush-edge (Innes, 1978). The wide dispersal of the ship rat in forested regions in New Zealand is in contrast to the situation in Great Britain and other countries where it is generally confined to cities and ports.

The ship rat is very well adapted to New Zealand conditions. It is not seriously affected by competition from the Norway rat, as is the case in other countries, however the two species are seldom found in the same environment in New Zealand (Daniel,1969; Atkinson,1973). If both ship rats and Norway rats are present in the same built-up area, the ship rats generally occupy the roof and walls of buildings while the Norway rats live at ground level. The two species do not interbreed (Watson, 1959).

The ship rat is an arboreal species and nests in trees, although it can be ground-dwelling in the absence of other species (Best,1968; Daniel, 1972). Food in forested areas consists mainly of seeds and fruit in winter and arthropods in summer (Daniel,1973; Innes,1978). Animal matter is rarely consumed, which is in contrast to the diet of the Norway rat.

Movements of ship rats are limited and their home range in two forested areas in New Zealand has been calculated to be 0.05 and 0.17 ha (Daniel, 1972; Innes,1978). Home ranges in buildings or on farmland have not been determined.

The female breeding season lasts approximately six months in New Zealand (late spring to early autumn) which is in contrast to the absence of a defined breeding season in tropical countries. Males have mature sperm in the testes throughout the year (Innes, 1978). The female produces an average of six offspring per litter

and up to 30 offspring may be born per year (Watson, 1959; Bentley and Taylor, 1965).

Juvenile ship rats reach sexual maturity at two to three months of age and ovulation occurs approximately one month after perforation of the vagina (Bentley and Taylor, 1965; Daniel, 1972). The mean age at which perforation of the vagina first occurred in the study conducted by Bentley and Taylor (1965) was 68 days and the mean weight of these females was 83g. Daniel (1972) found that the minimum weight of a female with a performated vagina was 62g. and the 50% end point (50% of the population with the characterstic) in a study by Best (1972) was 80 grams. There is little information available on the onset of sexual maturity in male ship rats in New Zealand however Innes (1978) reported that the minimum weight of a male with scrotal testes was 82g.

Populations of ship rats are susceptible to marked fluctuations, both seasonally and annually (Beveridge, 1964; Daniel, 1972). Although capable of responding to favourable conditions very rapidly, densities on farmland and in forests in New Zealand are generally low. Brockie (1977) failed to see any rats during 34 hours spotlighting on dairy farms and densities of 1.7 and 2.8/ha have been calculated in forest areas by Daniel (1972) and Innes (1978). Low densitites are associated with predation by feral cats and stoats and the marked changes in seasonal availability of food. The annual mortality calculated from cage grid disappearances by Innes (1978) was 96% and Daniel (1972) calculated that the mean life expectancy from initial capture was 2.3 months for adult males and 4.5 months for adult females.

## Norway Rat (*Rattus norvegicus*)

This rat typically lives in close association with man and therefore it has a very patchy distribution compared to the widespread distribution of the ship rat (Wodzicki, 1950). Norway rats are generally found wherever food is easily obtainable, such as rubbish dumps and intensive pig and poultry farms. The Norway rat is also found in cities and suburbs (Watson, 1959) and occasionally in river beds in remote areas (Davidson, 1965).

Numbers on farmland are usually much lower than the number of ship rats (Brockie, 1977) but when favourable conditions for Norway rats exist, this species prevails over the ship rat (Daniel, 1969). However, the two species can live in the same environment if the population of Norway rats is low (Wodzicki, 1950).

The Norway rat lives and nests in burrows (Watson,1959). This rat is quite capable of living in cold and damp conditions and readily enters water. It is a very poor climber compared with the ship rat and therefore its diet is limited to food that is access-ible from the ground. This consists of grain, vegetables, carrion, a wide range of refuse and some food naturally-available in the environment (Wodzicki, 1950; Watson, 1959; Calhoun,1962). Cannabil-ism may occur (Calhoun,1962).

The female breeds all year round and, with an average litter size of eight, up to 40 offspring may be produced in a year (Watson 1959; Calhoun 1962). Sexual behaviour of young Norway rats is first observed at about 80 days of age, at which time the mean weight of males and females is approximately 240 to 175 g respect-ively (Calhoun,1962). The perforation of the vagina in females precedes sexual behaviour and this usually occurs in all females by 75 days of age (Calhoun,1962). Some females may not exhibit sexual behaviour until 115 days of age. Norway rats raised in the laboratory will breed at 12 to 16 weeks of age,however wild rats do not breed until at least 16 weeks of age and the majority do not breed until some weeks later. Norway rats exhibit overt sexual behaviour and, in a crowded environment, one female will often be mounted by a large number of males in a single night. Thus a high degree of sexual contact can occur between individuals in crowded situations (Calhoun,1962).

Although Norway rats are prolific breeders, there is a high annual mortality rate (Davis *et al*,1948; Wodzicki, 1950; Fitzgerald and Karl, 1977). Highest numbers occur in autumn and early winter but heavy predation by feral cats and mustelids and seasonal fluctuations in the availabiljty of food soon reduce this peak population. High local densitieε may be maintained in the presence of an abundant food supply and adequate refuge however.

When the population of Norway rats becomes high, social
interaction limits further growth, even in the face of an abundant
food supply and adequate refuge (Calhoun,1962).  In this situation
the growth of young animals in inhibited, there is no expression
of territoriality by males and the breeding success of females is
poor.  Overcrowding also encourages migration and migrating rats
may cover large distances.  This is in contrast to the situation
in a stable population where movements have been shown to be
restricted to as little as 33 metres (Davis *et al*, 1948).

The Norway rat is much more difficult to trap than the ship
rat (Watson, 1959; Calhoun,1962) as it's timid nature makes it
very wary of new objects placed in it's environment.

House Mouse (*Mus musculus*)

The house mouse is the only species of mouse present in New
Zealand and it is very widely dispersed (Wodzicki, 1950; Davidson,
1965; Daniel, 1972).  It is the most abundant rodent on dairy
farms (Brockie, 1977) and is commonly found in close association
with man as well as in remote and harsh habitats.  This is in
contrast to the situation in other countries where field and forest
habitats are occupied by different species of mice.  Lower numbers
are present in forests compared with farm environments.  The house
mouse has difficulty in co-existing with an uncontrolled population
of Norway rats (Taylor, 1975).

House mice nest wherever cover is available.  If the food
source is close to available shelter, the daily range of movement
may be very small (Watson, 1959).  The house mouse is a good climber
and can therefore utilize a variety of food sources, both in build-
ings and in natural environments.  Mice populations can reach high
densities when an artificial food supply is available (Wodzicki,1950).
Howell (1954) found that the home range of house mice in an over-
grown field was 0.67 ha.

Mice breed throughout the year, although the main breeding season
occurs in spring and summer (Fitzgerald and Karl, 1977).  An average
of six offspring are produced per litter and five litters may be

produced in one year.  Breeding is proportional to food supply (Watson, 1959).

There is very little information available on growth rates and time of onset of sexual maturity in free-living house mice.  Unsubstantiated reports have put the time of onset of sexual maturity as being sometime after four weeks of age, at which time wild mice have been reported to weigh 18 - 20 g.(Lane-Petter, 1976). Theiler (1972) found that the vagina perforates and oestrus cycles begin in the laboratory mice at six weeks of age and the first successful mating can occur at eight weeks.  The mean weight of laboratory-reared mice at eight weeks is 25 g. and at ten weeks is 27 g. (Theiler, 1972).  It is therefore doubtful that wild mice reach a weight of 18 - 20 g. by four weeks of age, or that sexual maturity may be reached by this time.  Full size in laboratory mice is achieved by five months of age and mature males are heavier than mature females (Theiler, 1972).

Dramatic fluctuations occur in the population density of mice (Fitzgerald and Karl, 1977).  In a forest environment these are due to variations in seeding and the success of the summer breeding season.  Mice populations are also heavily predated by cats, mustelids and birds (Wodzicki,1950; Fitzgerald and Karl, 1977). There is no published information on population densities or movements in New Zealand.

## CARNIVORA

There are three species of Mustelidae present in New Zealand.

### Ferret *(Putorius putorius)*

The ferret is the largest of the Mustelidae group and it is present in low numbers and has a restricted distribution. (The New Zealand ferret is the variety with polecat-like markings). Ferrets are found on farmland and occasionally in scrub however they are not found in wet forest(Wodzicki, 1950; Marshall, 1963). The  distribution of the ferret is in part related to the distribution of rabbits, which form a major part of its diet (Wodzicki,1950; Marshall, 1963).

Ferrets predate on rabbits, rodents and birds and they also eat carrion. They are unable to climb trees and are more easily trapped than stoats and weasels.

The female ferret is multiparous and may have up to four litters each year. Up to 14 offspring may be born in each litter, however the neonatal mortality rate is high (Wodzicki, 1950). Very little is known about the population density or movements of ferrets in New Zealand.

### Stoat *(Mustela erminea)*

The stoat is widely distributed and is the most successful of the mustelids to adapt to New Zealand conditions. This species is relatively common on farmland and it is also found in scrub and on the forest edge (Wodzicki, 1950). High numbers of stoats appear to be correlated with a high density of rabbits in the same envirionment (Marshall,1963).

Stoats feed on rabbits, rodents, birds, reptiles, invertebrates and carrion. They are less of a scavenger than the ferret (Marshall, 1963). They are good climbers and often hunt during the day which is in contrast to the nocturnal nature of the other mustelids (Wodzicki,1950). Nests are made in logs, derelict farm buildings and disused rabbit warrens.

The female stoat has two litters each year and up to ten offspring may be born in each litter. The main breeding season is in spring and early summer.

There is very little published information on the population dynamics of stoats in New Zealand, however they are generally thought to be more abundant than ferrets or weasels.

### Weasel *(Mustela nivalis)*

This mustelid is rare in New Zealand and has a very local distribution (Wodzicki 1950; Marshall, 1963). The diet of the weasel is based mainly on mice and young rats, although it will

attack rabbits. Little is known of the biology of this species.

## Feral cat (*Felis catus*)

Many cats were liberated in remote farmland areas in the late 19th Century to control rabbits and these animals formed the basis of a widely distributed, but low density, feral cat population (Davidson, 1965). The feral population is constantly reinforced by domestic cats.

Feral cats feed on rabbits, birds, rodents and carrion (Wodzicki 1950) and have been blamed for the depletion of many native bird species. Cats may be a significant factor in the control of rodents on farmland.

The survival rate of kittens born in the wild is thought to be very low (Wodzicki, 1950) and this is the probable reason why the population density remains low. Environments which support moderate rabbit populations appear to support higher numbers of feral cats than environments where rabbits are rare (Davidson, 1962).

## INSECTIVORA

## Hedgehog (*Erinaceus europaeus*)

The hedgehog was introduced from Europe in the late 1800's and New Zealand is the only country to which this species has been successfully introduced (Brockie, 1975b). It is widely distributed and is common on farmland (especially dairy farms) and in suburban areas (Smith, 1964; Parkes, 1972). Small numbers are also found in forest regions (Brockie, 1975b).

The hedgehog is nocturnal and hibernates during the winter, however some remain active throughout the year. It's diet consists of insects, mice and also eggs of ground-nesting birds (Wodzicki, 1950; Campbell, 1973), and Brockie and Till (1977) state that hedgehogs locate food in pasture and under leaves by constant sniffing with their nose held close to the ground. Nests are made under buildings, hedges and tree stumps.

Female hedgehogs breed in spring and early summer and may have up to three litters in a year (Wodzicki, 1950. Brockie, 1975b). Litter size varies from two to eight.

The limiting factors on hedgehog numbers are availability of food, availability of weatherproof over-wintering sites and the presence of the mange mite (*Caparinia tripilis*) (Campbell, 1973; Brockie, 1975b). The mange mite can be a significant factor in increasing the annual mortality rate of hedgehog populations. The hedgehog does not suffer from predation to any extent, although young may be eaten by ferrets and stoats.

Absolute population densities or hedgehogs have not been fully determined. Campbell (1973) reported that more than 100 animals visited an eight hectare plot on a dairy farm during a two and a half year period. He estimated that the population density was between four and eight per hectare on this farm. The relatively high population density in New Zealand is probably due to a long summer breeding season and an absence of competition for food by other insectivorous species.

The only other small mammalian species present in New Zealand are the rabbit *(Oryctolagus cuniculus)* and the hare *(Lepus europaeus)*. Only small numbers of these species were examined in the present study.

## METHODS OF CAPTURE

A variety of methods were used to capture the animals investigated in the present study. The majority of effort was on the capture of rodents as it was considered that these animals were likely to be the most important reservoirs of leptospirosis.

Initial attempts to capture rats were based on the use of snap traps (Plate 11.1). These were baited with carrot and peanut butter (Innes, 1978) and placed at stategic positions on the ground for the capture of Norway rats, and on ledges and in roofs of buildings for

the capture of ship rats. Snap traps killed the animals that were caught and therefore blood was not available for serological examination. Rats caught in snap traps placed on the ground were sometimes eaten by predators and this also limited the effectiveness of the technique.

Cage traps proved to be more effective for the capture of rats (Plate 11.2). These traps were set on the ground and pre-baited with carrot and peanut butter for three days before setting. One end of the trap was covered in and filled with wood shavings to provide warmth for trapped rats, as without this protection ship rats often died overnight in cold weather. Cage traps were an effective method for the capture of ship rats both on farms and in forest areas. Only limited numbers of Norway rats were caught in these traps and this was attributed to the wary nature of this species. As rats were captured alive, both blood and kidneys were available from trapped animals. The rats were transferred to a plastic bag and anaesthetised with ether which was injected into the bag. Blood was collected by either cardiac puncture or by severing the jugular vein, and kidneys were cultured immediately after death.

On some occasions, ship rats were caught in possum traps during the possum survey work. Rats caught in this manner were dead and therefore blood was not available. Some were eaten by predators. Norway rats were never caught in possum traps.

The most successful method of obtaining Norway rats was night-shooting. This method was restricted to locations where there were high population densities, such as rubbish dumps and grain stores. The rats were shot with 0.22 calibre birdshot and this ammunition was preferable to solid shot as it generally did not penetrate the abdominal cavity and therefore contamination of kidneys was avoided. Blood samples were collected immediately after shooting by opening the chest and removing a sample from the ventricles (which were often still beating) or the thoracic cavity. The rats were cultured in the laboratory the following morning. Using this method, up to 30 Norway rats could be sampled in one night from locations that had high densities. Spotlighting also allowed a subjective estimation of population density to be made.

Plate 11.1. : Ship rat captured in a snap trap.

Plate 11.2. : Cage trap for capture of rats.
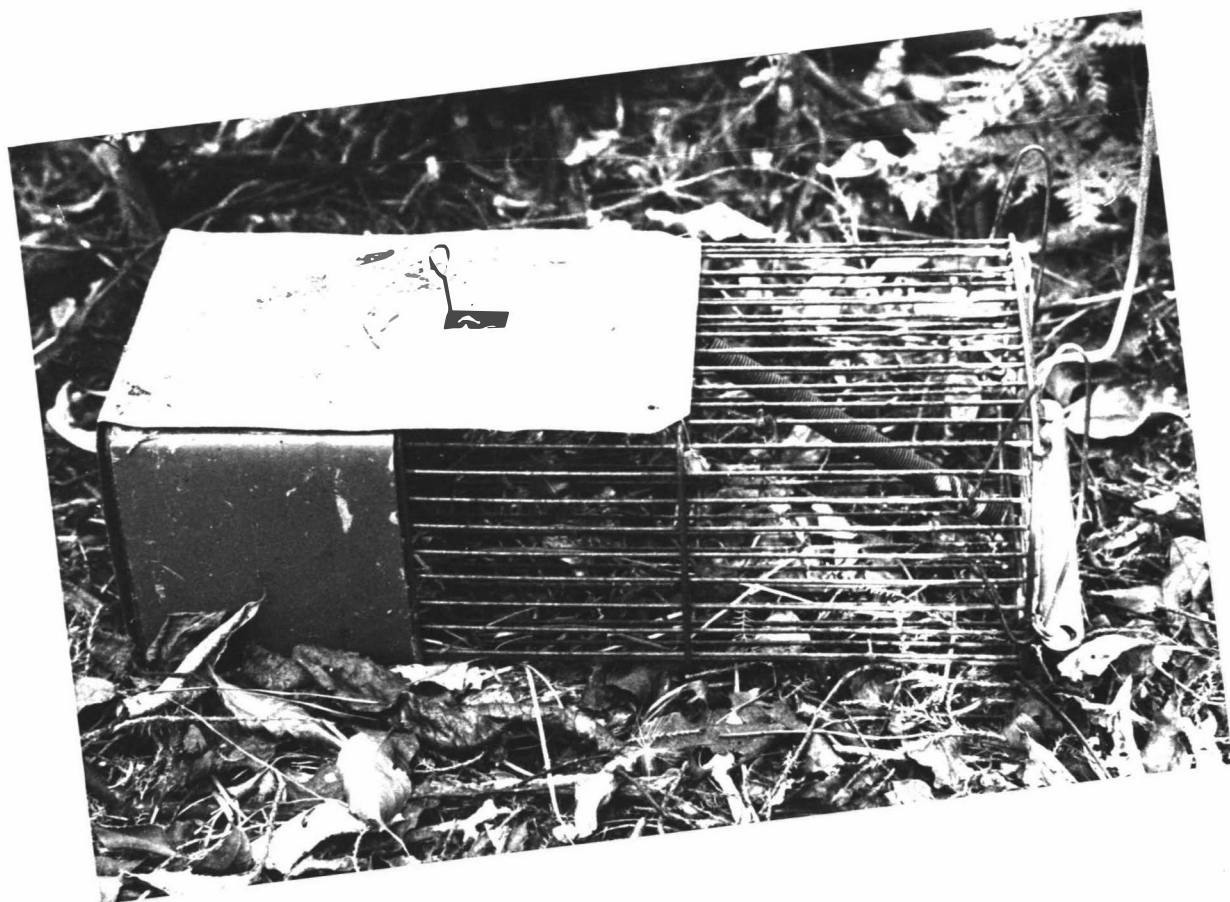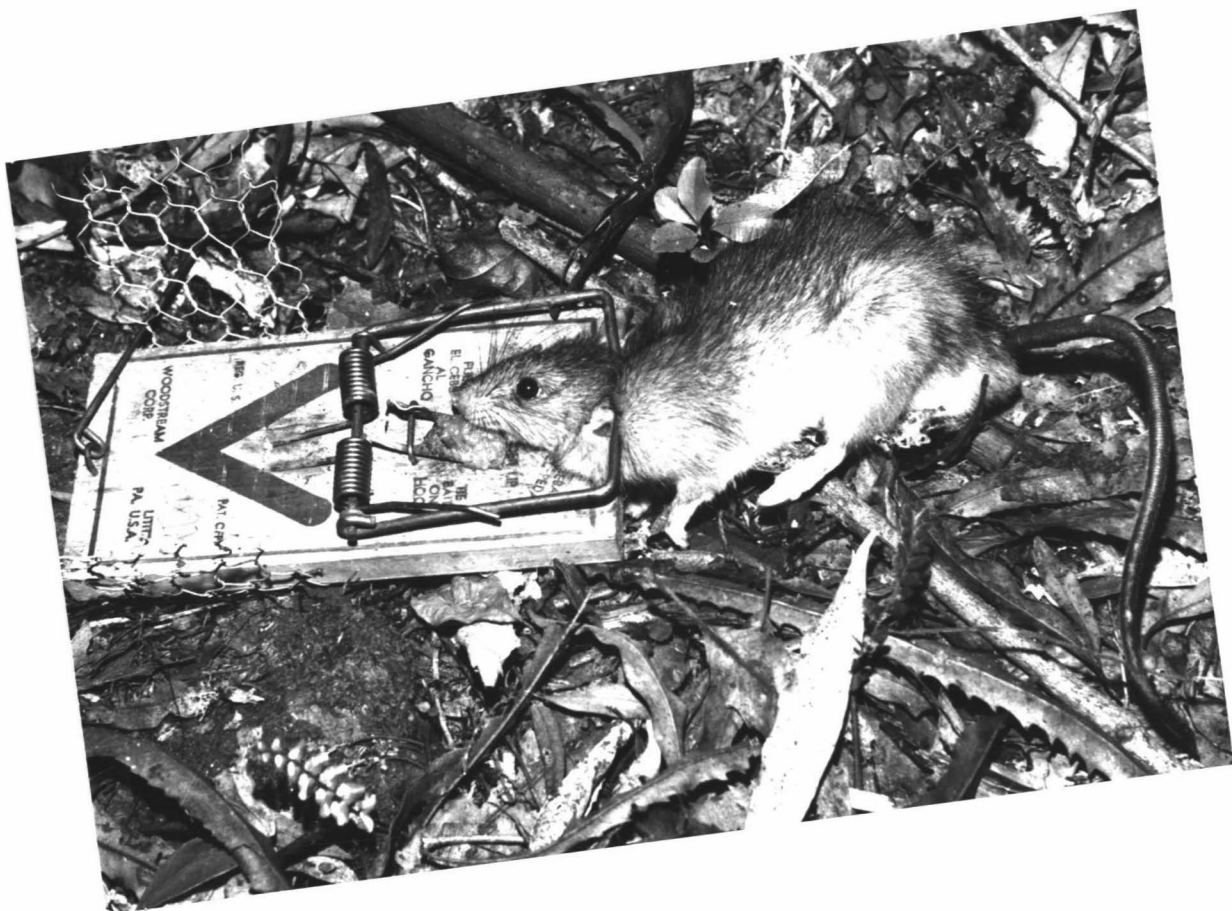
Ship rats were never seen by spotlighting. It appeared that the ship rat was excluded from environments that supported high densities of Norway rats.

Mice were captured by snap traps and by cage traps. Snap traps were only of limited use for the capture of mice. No blood was available and often mice were caught across the abdomen which caused effusion of fluid within the abdominal cavity. Kidney cultures from mice caught in this way were often contaminated.

Cage traps [a] (Plate 11.3) were eminently suitable for the live capture of mice. These traps automatically reset themselves after a mouse is caught and on two occasions five mice were captured in the same trap in one night. No bait was required and mice already captured appeared to attract orher mice. Their use was restricted to buildings however as only three mice were caught in these traps in the forest. (This was probably due to the low density of mice, due to limitations of food supply, in such areas).

Mice caught alive were transferred to a plastic bag and anaesthetised in the same way as rats. Blood samples were obtained by severing the major blood vessels in the neck. Kidneys were cultured immediately after death.

The mustelids gathered in the present survey were mostly captured in possum traps during possum surveys. As they were trapped alive, both blood and kidney samples could be obtained. A specific attempt to trap ferrets and stoats on a dairy farm, using possum traps baited with tinned cat food, resulted in the capture of only one ferret and one stoat. This was probably a reflection of the low numbers present. During a possum survey at White Rock in March 1976, a ferret was caught in a possum trap in a field in which a shepherd had seen several ferrets. Concentrated trapping with possum traps within a 300 m radius of the first capture resulted in four more ferrets being trapped. No ferrets were captured in possum traps in other locations on the farm and it is possible that the captured animals represented a localised focus.

[a]"Katch All" Havahart,Ossining,N.Y, U.S.A.
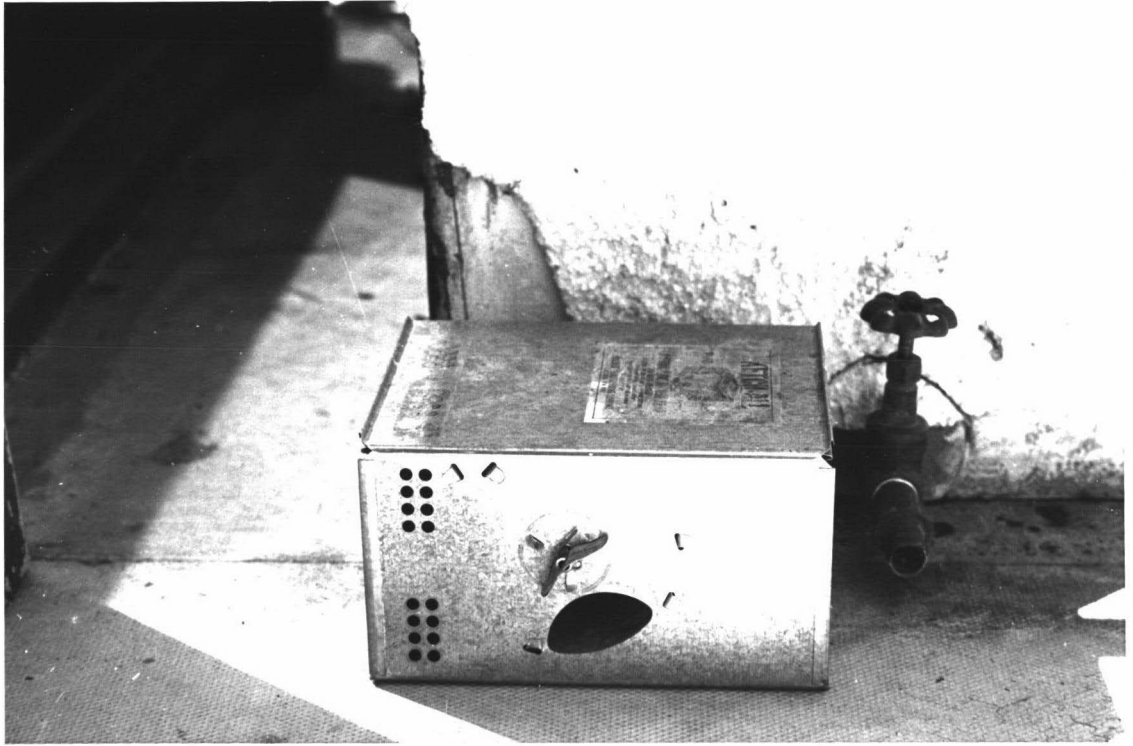
Plate 11.3. : 'Katch-All' automatic mouse trap.

A small number of ferrets and stoats were shot during night shooting operations. Two live weasels were captured in a coastal wildlife reserve.

Hedgehogs were captured by hand at night using spotlights. Occasionally hedgehogs were caught in possum traps set under farm shelter belts or on the edge of a forest. As hedgehogs were captured alive, blood and kidneys were available from all animals.

Feral cats were collected by shooting during night-shooting operations for possums. Some cats were also shot on rubbish dumps at night. Blood as well as kidneys were collected from these animals.

Only limited numbers of small mammals other than possums and rodents were captured, however examination of these species provided some preliminary information on leptospirosis in the less common free-living animals sharing common environments with possums and rodents in New Zealand.

CHAPTER XII

LEPTOSPIROSIS IN RATS

INTRODUCTION

Rats have been shown to be carriers of leptospirosis throughout the world (Chapter I) and are considered to be important reservoirs of infection for domestic livestock and man. The two species of rats present in New Zealand, the Norway and ship rat, have a cosmopolitan distribution and many different leptospiral serovars have been isolated from these species in different countries (Anon, 1966; Anon, 1975). The Norway rat is regarded to be the maintenance host for Icterohaemorrhagiae serogroup leptospires in many countries and is an occasional carrier of other serovars (Babudieri, 1958; Alexander *et al*, 1963; Michna and Ellis, 1974; Fennestad and Borg-Petersen, 1972; Mailloux, 1975; Hanson, 1976). The ship rat appears to be a less frequent carrier of leptospires (Emanuel *et al*, 1964; Torten *et al*, 1970) and several surveys have shown this species to be free of infection in some biotopes (Kirschner and Gray, 1951; Clark *et al*, 1966; Fennestad and Borg-Petersen, 1972).

Investigations of rats in New Zealand have revealed the presence of at least two serovars. Kirschner and Gray (1951) found serological evidence of Icterohaemorrhagiae serogroup infection in 8 of 53 urban Norway rats (15%). Isolates from this serogroup were recovered from two rats from Dunedin. Forty-seven urban ship rats were serologically and bacteriologically negative. A survey of 62 Norway rats and 121 ship rats from Wellington city by Blakelock and Allen (1956) revealed no evidence of leptospiral infection, as was the case in 15 rats (presumably Norway rats) caught around a piggery by Buddle and Hodges (1977). Brockie (1977) isolated leptospires of the Icterohaemorrhagiae serogroup, which he assumed to be serovar *copenhageni*, from 5 of 79 Norway rats (6%) collected from mainly rural areas in the North Island. These isolates all came from rural rubbish tips near Tirau and Morrinsville. Eight isolates of the Ballum serogroup (10%) were isolated from Norway rats from the Hawera rubbish tip. This worker also isolated leptospires of the

Ballum serogroup from 4 of 16 ship rats (25%) from mainly rural areas. Therefore, although both Norway and ship rats may be infected with leptospires in New Zealand, the reported prevalence of infection has been relatively low. This is in contrast to the situation in Norway rats in some other countries, where the prevalence of Icterohaemorrhagiae serogroup infection may be more than 50% (Babudieri, 1958; Schnurrenberger *et al*, 1970; Mailloux, 1975).

The following chapter describes an investigation of leptospirosis in rats in the southern half of the North Island of New Zealand. New Zealand provides a unique situation in which leptospires in rats can be investigated, in that there are only two species of rat present, very few other free-living species share a common environment with rats and only three serovars are known to be present in free-living animals. Hence the type ecosystem for a serovar (maintenance ecosystem for a particular infectious agent in nature) can be defined more easily than is the case in many other countries where there are a large number of serovars and free-living species present in a particular ecosystem. In addition, rats in New Zealand occupy a variety of biotopes and therefore leptospirosis can be studied in natural and syanthropic (man-influenced) nidi.

MATERIALS AND METHODS

Collection of rats

Rats were collected from a variety of locations representing different biotopes. The methods of collection have been described in Chapter XI. Collection methods were not designed to trap only one species in any one location sampled, however the differences in distribution and biology of the Norway and ship rat, as discussed in Chapter XI, were reflected in the collection results. Trapping success was determined by the calculation of trap-night averages (one trap set for one night equals one trap-night). These averages were used as indices of relative abundance of a species in different locations. Night-shooting also allowed indices of relative abundance to be calculated for rats inhabiting different rubbish tips. These indices were calculated from a subjective estimate of population density, the number of rats shot in 1.5 man-hours and the number of

rats counted in a 360° sweep of a spotlight made over two minutes. No figures are available on the population density of free-living species on farmland in New Zealand.

The farm locations that were sampled have been described in Chapter IV. Snap traps and cage traps were set in shelter belts and bush in gullies on White Rock and Otawhao Farms. Rats were also caught in possum traps on these farms during possum survey work. Rats captured at Linton were trapped in and around piggery buildings and a farm grain store.

The two forest locations that were trapped were both within 12 km of Massey University. The Manawatu Gorge Forest is a mixed podocarp-broadleaf forest and the trapline of snap and cage traps was situated approximately 25 m from the forest-pasture boundary. Trapping in the Tiritea Reserve, a forest area that serves as a water catchment for Palmerston North city, was conducted approximately 0.5 km from the nearest forest-pasture boundary. Traps on Kapiti Island were set in the bush close to the shoreline, an area where Norway rats were known to live ( J.Innes, pers. comm.).

Initial attempts to cage-trap rats on rubbish tips were largely unsuccessful and therefore all rats from these locations were taken by shooting. The Palmerston North and Feilding rubbish tips are large tips situated on suburban boundaries. Back-filling of the tip-face is conducted daily at Palmerston North but not at Feilding. Longburn rubbish tip is a small, privately-owned tip that serves the Longburn Freezing Works. The two rural rubbish tips, Kiwitea and Cheltenham, are small and cater for a low volume of refuse from farming districts.

The rats that were examined from urban Palmerston North were the results of only sporadic trapping and therefore trap-night averages were unavailable

## Serological and cultural examination

Sera from live-trapped rats were tested against *ballum*, *copenhageni*, *pomona*, *hardjo* and *tarassovi* antigens according to the

serological methods described in Chapter III.  The minimum serum dilution tested was 1:12.  A sample of 40 Norway rat and 11 ship rat sera were also tested against *canicola, pyrogenes, grippotyphosa, bataviae, autumnalis, australis* and *biflexa* antigens.  Sera were not available from rats that were caught in snap traps nor from those that died in traps overnight.  Blood samples were not collected from Norway rats shot on the Palmerston North tip from the 20th to the 22nd September 1977 and rats shot at the Feilding tip from the 4th to the 9th September 1977 (26% of Norway rats sampled), as serological results from earlier samples had revealed a very low prevalence of titres.

The cultural method employed for kidneys from Norway rats was the same as that used for possum kidneys (see Chapter III), except that the media series without 5FU was replaced with a media series containing 400 µg 5FU/ml.  This was necessary because preliminary cultures of rat kidneys in media without 5FU resulted in 9 of 50 culture tubes being contaminated (18%), whereas only 4.5% of  possum kidney cultures in this media were contaminated.

The kidneys of ship rats could not be homogenised in the Stomacher as the amount of tissue was too small and therefore an aseptic syringe technique was used.  Whole kidneys were placed in the barrel of a sterile, five ml, disposable syringe and then forced through a 14 gauge by 0.5 inch needle into five mls of Stuart's basal medium (SBM).  This primary homogenate was then shaken vigourously.  The remainder of the cultural method for kidneys from ship rats was the same as that for Norway rats.  The kidneys of juvenile Norway rats were also homogenised using the syringe technique.

RESULTS

The results of trapping and shooting in different localities demonstrated that Norway and ship rats have different distributions in New Zealand (Table 12.1).  Norway rats were found on rubbish tips, around a piggery and grain store at Linton and also in suburban Palmerston North.  Except for Kapiti Island, this species was not found in environments that did not provide artificial food sources

TABLE 12.1: Trapping success of snap and cage traps in different locations for Norway and ship rats.

| Location | Snap traps | | | Cage traps | | | Total trap-night averages | |
|---|---|---|---|---|---|---|---|---|
| | trapnights | Norway rat | ship rat | trapnights | Norway rat | ship rat | Norway rat | ship rat |
| White Rock Farm** | 72 | 0 (0) | 1 (1.4)* | NT | – | – | 0 | 1.4 |
| Manawatu Gorge Forest *** | 108 | 0 (0( | 3 (2.8) | 48 | 0 (0) | 1 (2.1) | 0 | 2.6 |
| Tiritea Reserve | NT | – | – | 278 | 0 (0) | 11 (4) | 0 | 4 |
| Otawhao farm **** | 84 | 0 (0) | 2 (2.4) | 132 | 0 (0) | 4 (3) | 0 | 2.8 |
| Linton | 144 | 2 (1.4) | 1 (0.7) | 472 | 10 (2.1) | 4 (0.8) | 1.9 | 0.8 |
| Kapiti Island | 18 | 0 (0) | 0 (0) | 12 | 1 (8.3) | 0 (0) | 3.3 | 0 |
| Palmerston Nth city | NT | | | NT | | | NT | |

*      Trap night average, calculated for 100 trap nights

**      2 ship rats captured in possum traps

***      11 ship rats captured in possum traps

****      10 ship rats captured in possum traps

associated with the activity of man. Ship rats were trapped in
forest, farmland and suburban areas. This species was only found
to share a common biotope with Norway rats at Linton and in
suburban Palmerston North.

Different environments supported markedly different population
densities. Norway rats were present in large numbers on rubbish
tips, with the highest density being found at Feilding tip. Relative
indices of abundance of Norway rats at other tips, compared with
Feilding, are given in Table 12.9. No ship rats were seen at
rubbish tips.

Trapping success (trap-night averages) were used as a guide
to the relative population density of rats on farmland and in
forest areas (Table 12.1). Manawatu Gorge Forest and Otawhao Farm
had similar trap-night averages (2.6 and 2.8/100 trap-nights
respectively) and these figures can be equated to a moderate popu-
lation of ship rats under New Zealand conditions (J.Innes, 1978).
A high trap-night average for Tiritea Reserve indicated that a
high density of ship rats was present in this forest. The
population density of Norway and ship rats at Linton was low
(trap-night averages of 1.9 and 0.8 respectively).

A total of 232 Norway rats (Table 12.2) and 52 ship rats (Table
12.3) were collected and all were subjected to cultural examination.
Blood samples for serological examination were obtained from 155
Norway rats (67%) and 21 ship rats (40%).

The sex ratio of the Norway rats collected was 1.94:1 in favour
of males and this was significantly different from parity ($X^2 = 11.2$,
$P < 0.005$) (Table 12.5). The sex ratio of ship rats was 1.26:1 in
favour of males. This ratio was not significantly different from
parity ($X^2 = 0.34$, $P > 0.5$).

Serological results are presented in Table 12.4. Very few titres
were found in sera from both species of rats. Titres against *ballum*
antigen were detected in only 5 of 155 Norway rats (3%) and the
range of these titres was from 1:12 to 1:48. *Ballum* titres were

Table 12.2 : Collection data and isolation of leptospires of the
Ballum serogroup from Norway rats.

| Sampling location | Date | No. collected | No.Ballum serogroup isolates | Prevalence(%) |
|---|---|---|---|---|
| Palmerston North rubbish tip | 8.5.77 | 2 | 0 | 0 |
| | 12.5.77 | 6 | 0 | 0 |
| | 19.5.77 | 5 | 0 | 0 |
| | 30.5.77 | 3 | 0 | 0 |
| | 1.6.77 | 4 | 0 | 0 |
| | 14.6.77 | 4 | 1 | 25 |
| | 23.8.77 | 1 | 0 | 0 |
| | 3.10.77 | 4 | 0 | 0 |
| Longburn rubbish tip | 24.8.77 | 3 | 1 | 33 |
| | 25.8.77 | 1 | 0 | 0 |
| | 28.8.77 | 5 | 0 | 0 |
| | 2.9.77 | 10 | 0 | 0 |
| | 5.9.77 | 5 | 0 | 0 |
| | 20.9.77 | 12 | 3 | 25 |
| | 22.9.77 | 14 | 4 | 29 |
| | 5.10.77 | 3 | 0 | 0 |
| | 23.3.78 | 2 | 0 | 0 |
| Feilding rubbish tip | 7.9.77 | 24 | 14 | 58 |
| | 8.9.77 | 13 | 6 | 46 |
| | 9.9.77 | 21 | 12 | 57 |
| | 12.10.77 | 20 | 4 | 20 |
| | 13.10.77 | 4 | 0 | 0 |
| | 19.10.77 | 9 | 1 | 11 |
| | 2.3.78 | 28 | 15 | 54 |
| | 23.3.78 | 4 | 1 | 25 |
| Kiwitea rubbish tip | 4.10.77 | 6 | 1 | 17 |
| Cheltenham rubbish tip | 5.10.77 | 1 | 0 | 0 |
| Palmerston North City | 28.6.76 | 1 | 0 | 0 |
| | 22.3.77 | 1 | 0 | 0 |
| | 10.5.77 | 1 | 0 | 0 |
| | 9.6.77 | 1 | 0 | 0 |
| | 28.9.77 | 1 | 0 | 0 |
| Linton | 13.7.77 to 29.9.77 | 6 | 0 | 0 |
| | 30.9.77 to 16.10.77 | 6 | 0 | 0 |
| Kapiti Island | 3.4.77 | 1 | 0 | 0 |

Table 12.3 : Collection data and isolations of leptospires of the Ballum serogroup from ship rats.

| Sampling location | Date | No. collected | No. Ballum serogroup isolates | Prevalence (%) |
|---|---|---|---|---|
| White Rock Farm | 18.3.76–23.3.76 | 3 | 1 | 33 |
| Manawatu Gorge Forest | 3.6.76–18.6.76 | 15 | 5 | 33 |
| Tiritea Reserve | 23.6.76–28.9.76 | 11 | 1 | 9 |
| Otawhao Farm | 23.3.77 | 1 | 0 | 0 |
|  | 18.5.77 | 1 | 0 | 0 |
|  | 23.6.77 | 1 | 0 | 0 |
|  | 6.8.77–12.8.77 | 13 | 8 | 62 |
| Linton | 13.7.77–29.9.77 | 5 | 2 | 40 |
| Palmerston North City | 5.3.77 | 1 | 0 | 0 |
|  | 8.7.77 | 1 | 0 | 0 |

found in sera from 5 of 21 ship rats (24%) and the range of these titres was from 1:48 to 1:192.  Titres  against *pyrogenes* occurred in three Norway rats and two ship rats and two Norway rats had titres of 1:24 against *tarassovi*.  A titre of 1:12 against *canicola* was found in a male ship rat with a *ballum* titre of 1:96.  The low prevalence of titres in rats prevented a statistical analysis of differences in prevalence by age, sex and location being carried out.

Leptospiral isolates were obtained from 63 of 232 Norway rats (27%) and 17 of 52 ship rats (33%) (Tables 12.5 and 12.6).  All isolates were serotyped as belonging to the Ballum serogroup and one isolate from each species was typed by cross-agglutination absorption at the WHO Reference Laboratory at Atlanta, U.S.A. as being serovar *ballum*.

Isolates were recovered in many cases from seronegative rats (Table 12.4).  Leptospires were cultured from 44 Norway rats from which sera were examined and 39 of these isolations were made from sero-negative animals (89%).  Twelve culture-positive ship rats were examined serologically and eight of these animals were sero-negative (67%).  Only one animal, a male ship rat, was serologically positive to *ballum* but negative on culture.  The ratio of serological prevalence of *ballum* titres to bacteriological prevalence of those examined serologically was 3:28 for Norway rats and 24:57 for ship rats.

The prevalence of infection in Norway rats by sex is shown in Table 12.5.  No statistically-significant sex differences in bacteriological prevalences were found in rats from Longburn and Feilding tips ($Xc^2 = 0.02$, P> 0.75; $Xc^2 = 1.69$, P >0.1 respectively). Prevalences of infection in individual samples of Norway rats from other locations were too low for statistical analysis of sex differences.  When Norway rats from all locations were combined, 30% of males and 22% of females were shown to be infected.  This sex difference was non-significant ($Xc^2 = 0.83$, P >0.25).

The bacteriological prevalences by sex in ship rats are given in Table 12.6.  When ship rats from all localities were combined, 38%

Table 12.4 : Serological and cultural evidence of leptospiral
infection in Norway and ship rats.

| Species | Location | Sex | Weight (g) | Titre | | Culture | Isolate serogroup |
|---------|----------|-----|-----------|-------|---|---------|-------------------|
| Norway rat | Palmerston North tip | M | 480 | 1:24 | *tarassovi* | - | |
| | | M | 410 | 1:24 | *pyrogenes* | - | |
| | | M | 380 | 1:48 | *ballum* | + | Ballum |
| | | | | 1:24 | *tarassovi* | | |
| | | M | 415 | 1:24 | *pyrogenes* | - | |
| Norway rat | Feilding tip | F | 325 | - | | + | Ballum |
| | | M | 490 | _ | | + | Ballum |
| | | M | 315 | 1:24 | *ballum* | + | Ballum |
| | | F | 330 | - | | + | Ballum |
| | | F | 390 | - | | + | Ballum |
| | | F | 430 | - | | + | Ballum |
| | | F | 380 | 1:12 | *ballum* | + | Ballum |
| | | M | 445 | - | | + | Ballum |
| | | M | 425 | - | | + | Ballum |
| | | M | 440 | - | | + | Ballum |
| | | M | 460 | - | | + | Ballum |
| | | M | 330 | 1:12 | *ballum* | + | Ballum |
| | | M | 460 | - | | + | Ballum |
| | | F | 390 | - | | + | Ballum |
| | | M | 385 | - | | + | Ballum |
| | | M | 280 | NT | | + | Ballum |
| | | F | 280 | NT | | + | Ballum |
| | | M | 355 | NT | | + | Ballum |
| | | F | 340 | NT | | + | Ballum |
| | | M | 400 | NT | | + | Ballum |
| | | M | 390 | 1:48 | *pyrogenes* | - | |
| | | M | 430 | - | | + | Ballum |
| | | M | 195 | 1:48 | *ballum* | + | Ballum |
| | | M | 45 | - | | + | Ballum |
| | | F | 205 | - | | + | Ballum |
| | | M | 55 | - | | + | Ballum |
| | | F | 185 | - | | + | Ballum |

Table 12.4 cont.

| Species | Location | Sex | Weight (g) | Titre | | Culture | Isolate serogroup |
|---------|----------|-----|------------|-------|---|---------|-------------------|
| Norway rat | Feilding tip | M | 380 | * | – | + | Ballum |
| | | M | 405 | * | – | + | Ballum |
| | | M | 450 | * | – | + | Ballum |
| | | M | 345 | * | – | + | Ballum |
| | | M | 350 | * | – | + | Ballum |
| | | M | 395 | * | – | + | Ballum |
| | | M | 425 | * | – | + | Ballum |
| | | M | 440 | * | – | + | Ballum |
| | | M | 385 | * | – | + | Ballum |
| | | M | 360 | * | – | + | Ballum |
| | | M | 460 | * | – | + | Ballum |
| | | M | 475 | * | – | ∻ | Ballum |
| | | M | 380 | * | – | + | Ballum |
| | | M | 350 | * | – | + | Ballum |
| | | M | 475 | * | – | + | Ballum |
| | | M | 375 | NT | | + | Ballum |
| | | M | 410 | " | | + | Ballum |
| | | M | 515 | " | | + | Ballum |
| | | F | 530 | " | | + | Ballum |
| | | M | 315 | " | | + | Ballum |
| | | M | 405 | " | | + | Ballum |
| | | M | 505 | " | | + | Ballum |
| | | F | 345 | " | | + | Ballum |
| | | M | 340 | " | | + | Ballum |
| | | F | 365 | " | | + | Ballum |
| | | F | 335 | " | | + | Ballum |
| | | F | 400 | " | | + | Ballum |
| Norway rat | Longburn tip | M | 340 | – | | + | Ballum |
| | | F | 430 | NT | | + | Ballum |
| | | M | 360 | " | | + | Ballum |
| | | M | 455 | " | | + | Ballum |
| | | M | 305 | " | | + | Ballum |
| | | M | 360 | " | | + | Ballum |

Table 12.4   cont.

| Species | Location | Sex | Weight (g) | Titre | Culture | Isolate serogroup |
|---|---|---|---|---|---|---|
| Norway rat | Longburn tip | M | 440 | NT | + | Ballum |
|  |  | M | 385 | " | + | Ballum |
| Norway rat | Kiwitea tip | F | 500 | – | + | Ballum |
| Ship rat | White Rock Farm | M | 140 | 1:192 *ballum* 1: 48 *pyrogenes* | + | Ballum |
|  | Manawatu Gorge Forest | M | 150 | 1:96 *ballum* 1:12 *canicola* | + | Ballum |
|  |  | M | 145 | NT | + | Ballum |
|  |  | M | 170 | – | + | Ballum |
|  |  | F | 120 | NT | + | Ballum |
|  |  | M | 80 | – | + | Ballum |
| Ship rat | Tiritea Reserve | F | 185 | – | + | Ballum |
|  |  | M | 140 | 1:96 *ballum* | – |  |
| Ship rat | Otawhao Farm | M | 175 | – | + | Ballum |
|  |  | M | 155 | NT | + | Ballum |
|  |  | F | 180 | – | + | Ballum |
|  |  | F | 170 | NT | + | Ballum |
|  |  | M | 155 | – | + | Ballum |
|  |  | M | 150 | – | + | Ballum |
|  |  | F | 135 | NT | + | Ballum |
|  |  | M | 145 | 1:48 *ballum* | + | Ballum |
| Ship rat | Linton | M | 155 | – | + | Ballum |
|  |  | F | 135 | 1:96 *ballum* 1:24 *pyrogenes* | + | Ballum |

* tested against serovars *ballum* and *copenhageni* only.

Table 12.5 : Ballum serogroup leptospiral infection in Norway rats from all locations.

| Location | No. collected | Sex | | *ballum* titres | | | Ballum serogroup isolates | | | Bact.prevalence (%) | Sero* : bact.prev. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | M | F | M | F | total | M | F | total | | |
| Palmerston North tip | 29 | 20 | 9 | 1 | 0 | 1 | 1 | 0 | 1 | 3 | 1:1 |
| Longburn tip | 55 | 43 | 12 | 0 | 0 | 0** | 7 | 1 | 8 | 15 | − |
| Feilding tip | 123 | 74 | 49 | 3 | 1 | 4*** | 38 | 15 | 53 | 43 | 5:43 |
| Kiwitea tip | 6 | 3 | 3 | 0 | 0 | 0 | 0 | 1 | 1 | 17 | − |
| Cheltenham tip | 1 | 1 | 0 | 0 | − | 0 | 0 | − | 0 | 0 | 0 |
| Palmerston North City | 5 | 3 | 2 | NT | NT | NT | 0 | 0 | 0 | 0 | 0 |
| Linton | 12 | 8 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Kapiti Island | 1 | 1 | 0 | 0 | − | 0 | 0 | − | 0 | 0 | 0 |
| Total | 232 | 153 | 79 | 4 | 1 | 5 | 46 | 17 | 63 | 27% | 3.28 |

* ratio calculated for those rats from which sera examined

** 28/55 (51%) subjected to serological examination

*** 78/123 (63%) subjected to serological examination

Table 12.6 : Ballum serogroup leptospiral infection in ship rats from all locations

| Location | No. collected | Sex M | Sex F | *ballum* titres M | *ballum* titres F | *ballum* titres Total | Ballum serogroup isolates M | Ballum serogroup isolates F | Ballum serogroup isolates Total | Bact. prevalence(%) | *Sero : bact.prev. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| White Rock Farm | 3 | 1 | 2 | 1 | 0 | 1 | 1 | 0 | 1 | 33 | 1:1 |
| Manawatu Gorge | 15 | 10 | 5 | 1 | 0 | 1[a] | 4 | 1 | 5 | 33 | 1:3 |
| Tiritea Reserve | 11 | 5 | 6 | 1 | 0 | 1[b] | 0 | 1 | 1 | 9 | 1:1 |
| Otawhao Farm | 16 | 9 | 7 | 1 | 0 | 1[c] | 5 | 3 | 8 | 50 | 17:83 |
| Linton | 5 | 3 | 2 | 0 | 1 | 1[d] | 1 | 1 | 2 | 40 | 1:2 |
| Palmerston North City | 2 | 1 | 1 | NT | NT | NT | 0 | 0 | 0 | 0 | — |
| Total | 52 | 29 | 23 | 4 | 1 | 5 | 11 | 6 | 17 | 33 | 24:57 |

* ratio calculated for those rats from which sera examined.

[a] 5/15 (33%) subjected to serological examination.

[b] 3/11 (27%) subjected to serological examination.

[c] 6/16 (38%) subjected to serological examination.

[d] 4/5 (80%) subjected to serological examination.

of males and 26% of females were shown to be infected. This sex difference was non-significant ($Xc^2$ = 0.13, $P > 0.05$).

The percentage distribution of Norway rats of different weights collected at Feilding tip and the combined sample of Norway rats from tips other than Feilding are given in Table 12.7 and Figure 12.1. The majority of rats in both samples weighed between 300 and 450 g (56% and 66% respectively). Both samples had a similar percentage distribution of adult rats of different weights, thus indicating a similar population structure. The minimum weight at which Norway rats can be classified as being sexually mature is 150 g (see Chapter XI) and 19 rats from Feilding tip weighed less than this. Only one Norway rat obtained from tips other than Feilding weighed less than 150 g.

The prevalence of infection in Norway rats of different weights is given in Table 12.7 and Figure 12.1. Isolates were obtained from 2 of 19 Feilding rats weighing less than 150 g (11%). The single immature rat in the combined sample from tips other than Feilding was culturally negative. There was a marked difference in the prevalence of infection in sexually mature rats from Feilding compared with the combined sample of rats from other tips. All weight-groups of rats from Feilding had a higher prevalence of infection and, as the weight of sexually-mature rats increased, the prevalence of infection in each weight group increased until it reached 100% in the heaviest group of rats. The prevalence of infection in the combined sample of sexually-mature rats from tips other than Feilding did not show a continued increase as weight increased. The highest prevalence of infection (17%) occurred in those rats weighing 300 - 399g, and levels in heavier weight groups did not exceed 11% (Figure 12.1).

The percentage distribution of ship rats of different weights is given in Table 12.8 and Figure 12.2. The lowest weight at which ship rats can be classified as being sexually mature is 60g (see Chapter XI). All ship rats trapped in the present survey weighed more than this. The highest percentage of rats were in the 120 - 149g group (33%) and this group also had the highest prevalence of infection (47%). The prevalence of infection in heavier weight groups (150 - 179g and 180 - 209g) was 43% and 33%.

Table 12.7: Norway rats from rubbish tips by weight-range groups and number of Ballum serogroup isolations within each weight-range group.

| Location | Number of rats in each weight-range group | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | <50g | 50-99g | 100-149g | 150-199g | 200-249g | 250-299g | 300-349g | 350-399g | 400-449g | 450-499g | >500g |
| Feilding Tip | 5(4) | 8(7) | 6 (5) | 5 (4) | 7 (6) | 10 (8) | 21 (17) | 27(22) | 21 (17) | 10(8) | 3 (2) |
| Tips other than Feilding | 0 | 0 | 1 (1) | 1 (1) | 5 (6) | 4 (4) | 12 (13) | 23(25) | 25 (28) | 11(12) | 9(10) |
| | Number of Ballum serogroup isolations within each weight-range group | | | | | | | | | | |
| Feilding Tip | 1(20) [**] | 1(13) | 0 | 2 (40) | 1 (14) | 2(20) | 10 (48) | 14(52) | 12(57) | 7(70) | 3(100) |
| Tips other than Feilding | – | – | 0 | 0 | 0 | 0 | 2 (17) | 4(17) | 2 (8) | 1( 9) | 1 (11) |

* % distribution of weight-range groups

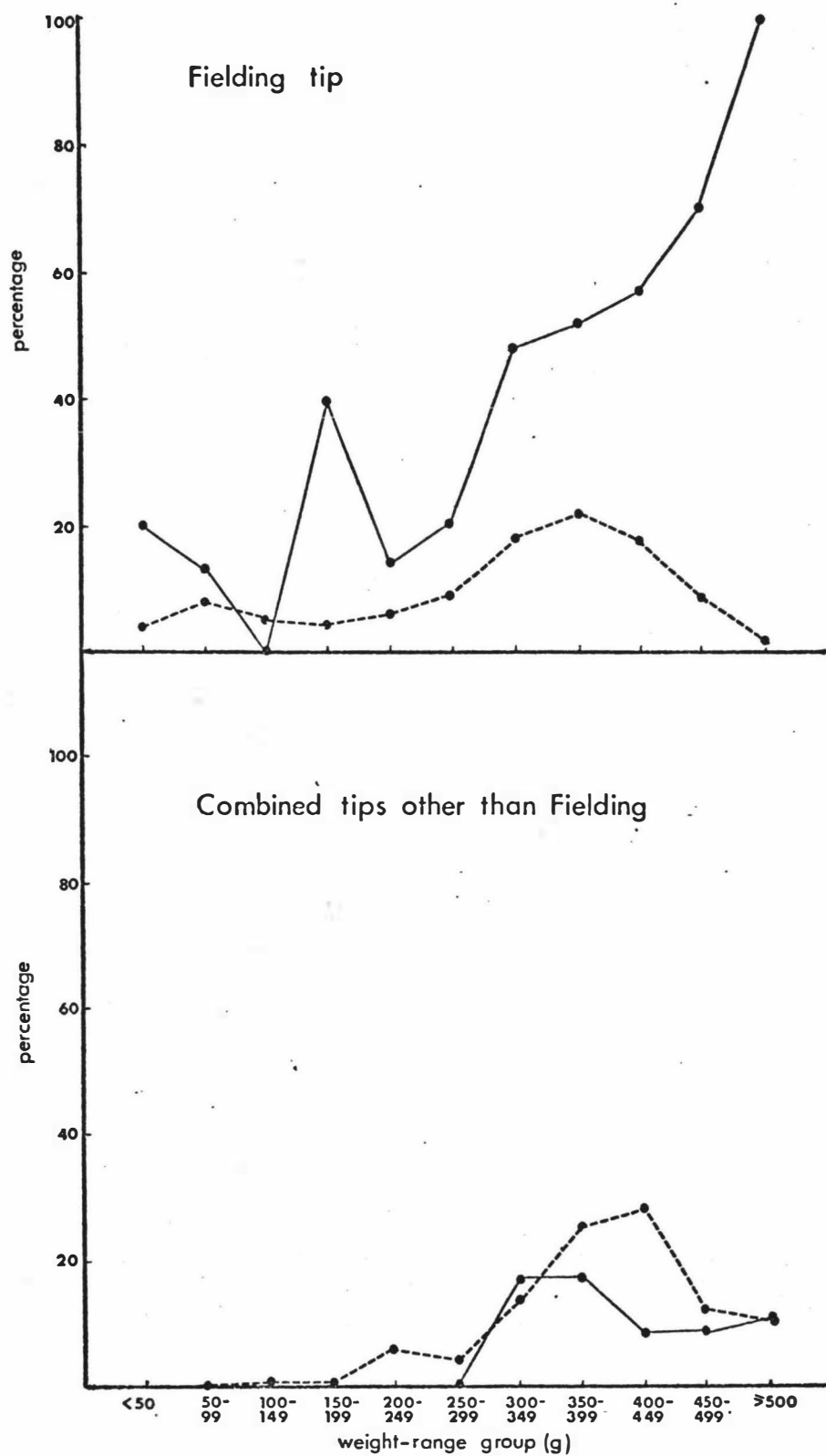** prevalence of Ballum serogroup infection within each weight-range group.

Figure 12.1. : Percentage distribution of weight-range groups
of Norway rats from rubbish tips and the prevalence
of Ballum serogroup infection in different weight-range
groups.

—— prevalence of Ballum serogroup infection within weight-range groups.
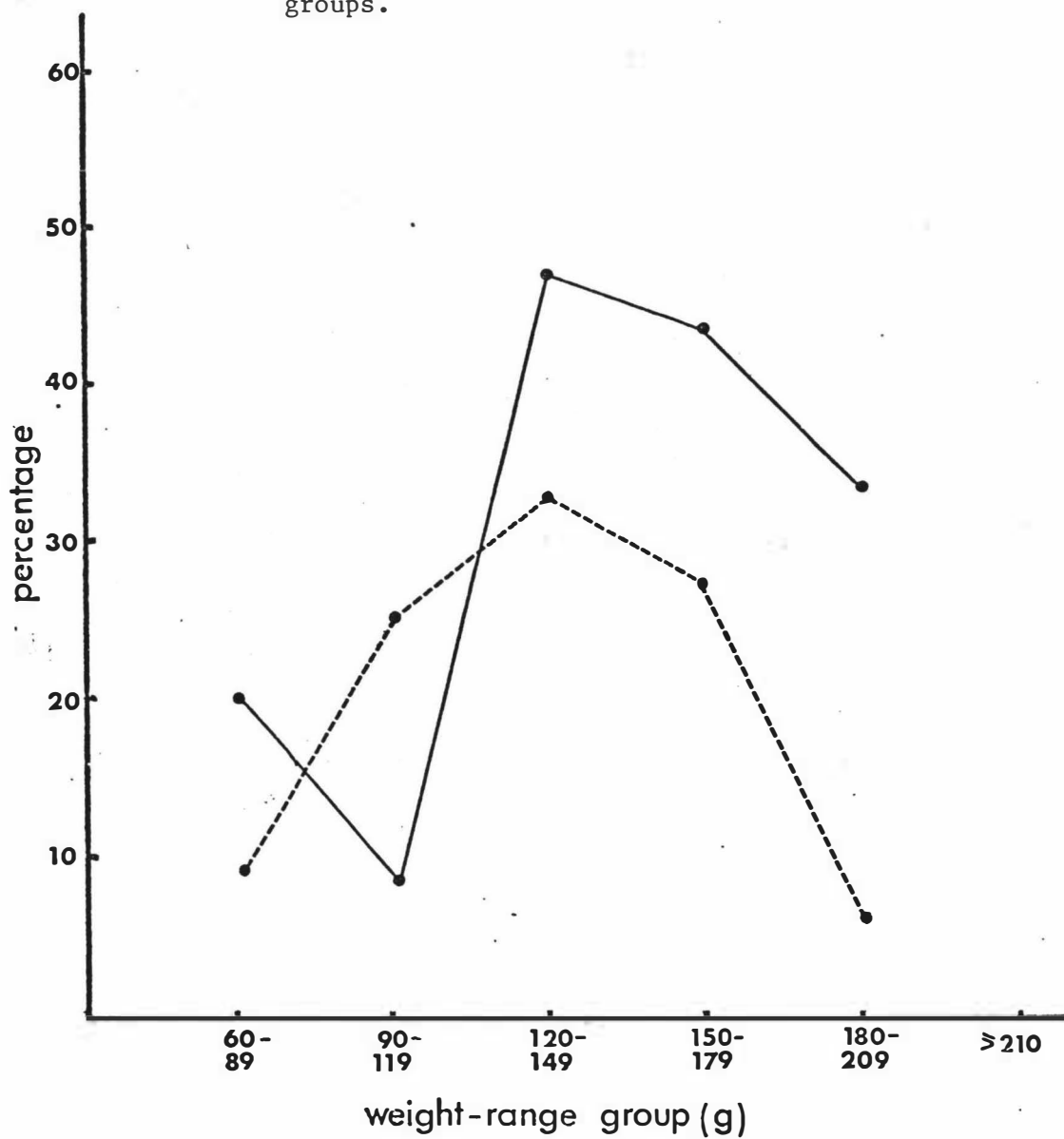---- percentage distribution of weight-range groups.

Table 12.8 : Ship rats by weight-range group and number of Ballum serogroup isolations within each weight-range group.

| | Weight-range groups (g) | | | | | | |
|---|---|---|---|---|---|---|---|
| | < 60 | 60 - 89 | 90 - 119 | 120 - 149 | 150 - 179 | 180 - 209 | ⩾ 210 |
| No. of rats within each weight-range group | 0 | 5 (9)[*] | 13 (25) | 17 (33) | 14 (27) | 3 (6) | |
| No. of isolations within each weight-range group | − | 1 (20)[**] | 1 ( 8) | 8 (47) | 6 (43) | 1 (33) | |

\* % distribution of weight-range groups

\** prevalence of Ballum serogroup infection within each weight-range group.

Figure 12.2. : Percentage distribution of weight-range groups
of ship rats and the prevalence of Ballum
serogroup infection in different weight-range
groups.

—— prevalence of Ballum serogroup infection within weight-
--- range groups.

--- percentage distribution of weight-range groups.

There was a marked difference in the prevalence of infection in Norway rats from different locations (Table 12.5). Rats from Feilding tip had a bacteriological prevalence of 43%. If immature rats were excluded from the sample, the bacteriological prevalence was 49%. Norway rats from Kiwitea, Longburn and Palmerston North tips had bacteriological prevalences of 17%, 15% and 3% respectively. No isolates were cultured from Norway rats from Palmerston North City or Linton. The two rats from Cheltenham tip and Kapiti Island were also negative. The difference in prevalence of infection in Norway rats from different tips was highly significant ($x^2$ = 26.72, P <0.001).

The differences in prevalence of infection in ship rats from different locations were much less pronounced than was the case for Norway rats (Table 12.6). Samples of ship rats trapped in rural areas (White Rock Farm , Otawhao Farm and Linton ) had bacteriological prevalences of 33%, 40% and 50% respectively. These differences were non-significant ($Xc^2$ = 0.26, P> 0.75). Ship rats from the Manawatu Gorge Forest and the Tiritea Reserve had bacteriological prevalences of 33% and 9% respectively. This difference was non-significant, due to the small numbers in the samples ($Xc^2$ = 0.96, P > 0.25). Two ship rats from suburban Palmerston North were culturally negative.

A consideration of the relative indices of abundance of rats from different locations and the prevalence of infection provides some important epidemiological data on Ballum serogroup leptospirosis in rats in New Zealand. The rubbish tips that were sampled represented similar biotopes for the Norway rat and there was a strong correlation between the subjective indices of relative abundance (Table 12.9) and the prevalence of infection in Norway rats from different tips ( r = 0.96, P< 0.01). The regression of prevalence of Ballum serogroup infection in Norway rats from different tips on relative abundance of rats was y = 41.2x + 1.96. This was also statistically significant (t = 4.83, P< 0.05). Thus there was an 8.2% increase in the prevalence of infection for each 0.2 unit increase in the relative abundance of tip rats (Figure 12.3). The Norway rat populations at Linton and in suburban Palmerston North were of unknown relative abundance to tip rats, as trapping was used as the method of capture.

Table 12.9 : Subjective estimation of relative abundance of Norway rats on rubbish tips *

| Location | Size | Density estimates | | | | | | Combined estimate of relative abundance |
|---|---|---|---|---|---|---|---|---|
| | | Subjective | (ratio) | Max.no. shot / 1½hrs. | (ratio) | Spotlight count, 360° in 2 min. | (ratio) | |
| Feilding | large | xxxxxx | (1.0) | 30 | (1.0) | 45 | (1.0) | 1.0 |
| Longburn | small | xxx | (0.5) | 12 | (0.4) | 8 | (0.18) | 0.36 |
| Kiwitea | small | xx | (0.33) | 6 | (0.2) | 3 | (0.07) | 0.21 |
| Palmerston North | large | x** | (0.17) | 6 | (0.2) | 2 | (0.04) | 0.14 |

* estimated on 4.10.77 and 5.10.77

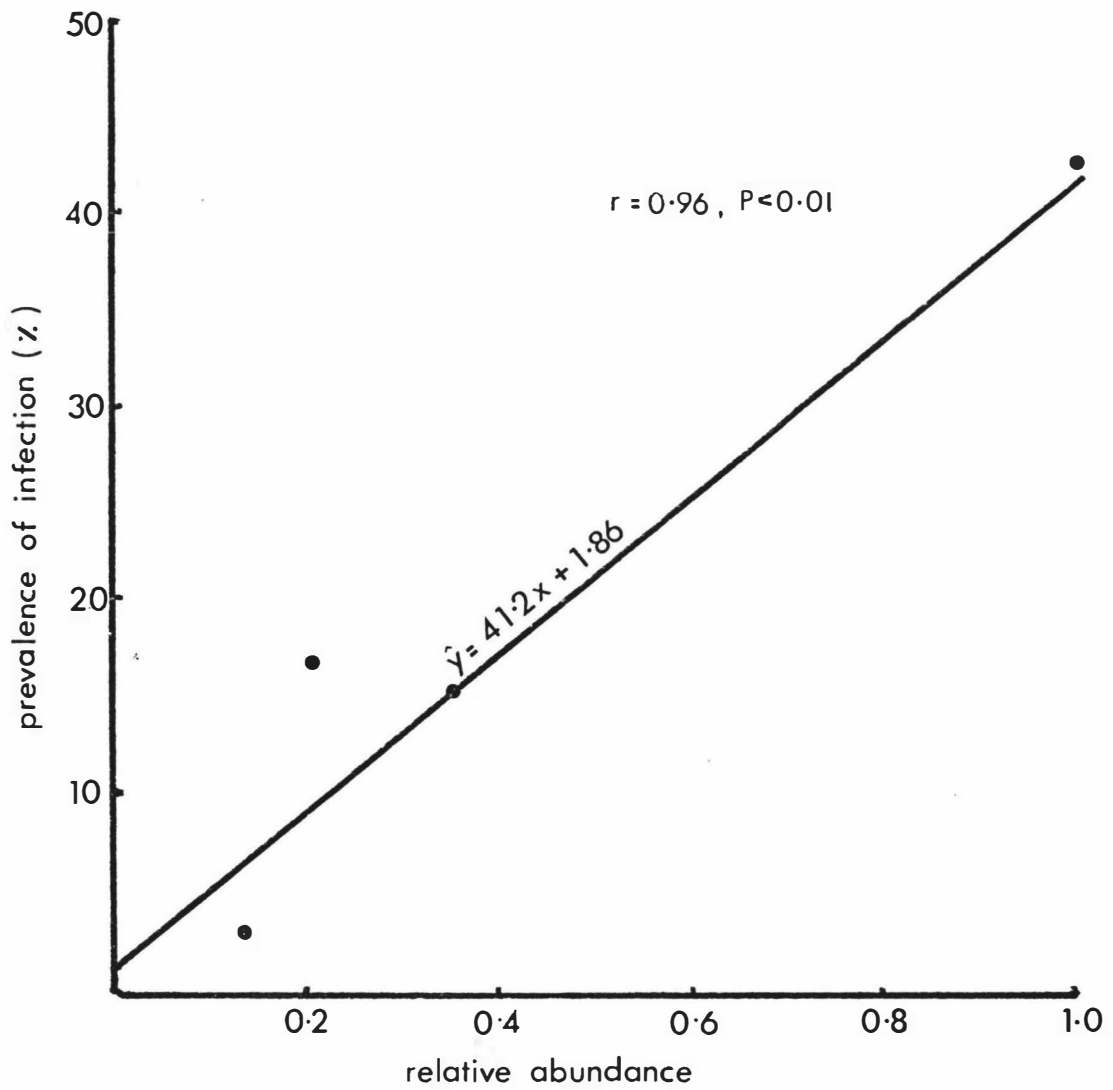** a subjective assessment when shooting first started (May,1977) would have been xx.

Figure 12.3. : Regression of prevalence of Ballum serogroup
infection in Norway rats on subjective estimate
of relative abundance.

Relative indices of abundance of ship rats were calculated on the ratio of trap-night averages in different locations relative to the trap-night average of ship rats caught in the Tiritea Reserve (Table 12.10). The correlation between relative abundance of ship rats and prevalence of infection was non-significant ($r = 0.56$, $P > 0.05$), as was the regression of relative indices of abundance on prevalence of infection ( $y = 26.7x + 48.49$; $t = 1.16, P > 0.2$). Thus the prevalence of Ballum serogroup infection in different populations of ship rats was not associated with population density.

The Ballum serogroup organisms isolated in the present survey grew very vigorously in artificial media. Leptospires were often able to be detected as soon as one week after inoculation and 57 of the 80 isolates obtained (71%) grew in all culture tubes inoculated from one kidney. The contamination rate of media containing 200 µg 5FU/ml was 8%. This was higher than that for possum kidneys (4.5%) and was attributed to the different methods of collection for rats compared with possums. The contamination rate of media containing 400 µg 5FU/ml was 6%.

## DISCUSSION

Night-shooting proved to be a very efficient method for the collection of Norway rats when they were present in moderate to high population densities. The difficulty in trapping Norway rats has been referred to earlier and this is well illustrated by Brockie (1977), who reported a trap-night average of only 1:17 on two rubbish tips where there were large numbers of rats. (The trap-night average for Norway rats on dairy farms was 1:791). It is unknown whether the high percentage of male relative to female rats collected by shooting was due to sampling bias or a reflection of the true sex ratio in rubbish tip rat populations.

Trapping success for ship rats was similar to that reported by New Zealand workers investigating the biology of this species in forest environments (Daniel, 1972; Innes, 1978) and also supported the information given in Chapter XI on the distribution of the Norway and ship rats in New Zealand. The ship rat was trapped in all biotopes sampled except that where there was direct competition with large

Table 12.10 : Relative abundance of ship rats in
all locations sampled.

| Location | Prevalence of infection (%) | Trap-night average | Relative indices of abundance * |
|---|---|---|---|
| Tiritea Reserve | 9 | 4.0 | 1.0 |
| Otawhao Farm | 50 | 2.8 | 0.7 |
| Manawatu Gorge Forest | 33 | 2.6 | 0.65 |
| White Rock Farm | 33 | 1.4 | 0.35 |
| Linton | 40 | 0.8 | 0.2 |

* calculated relative to Tiritea Reserve.

numbers of Norway rats. Norway rats were restricted to syanthropic foci and the only biotopes where Norway and ship rats were found together were suburban Palmerston North and an intensive farming environment at Linton. Rat numbers in both these environments appeared to be low. It is therefore probable that there is only a minimal opportunity for transfer of leptospirosis between these species under New Zealand conditions.

Serological examinations revealed Ballum serogroup titres in 3% of Norway rats and 24% of ship rats, with GMT's of 1:24 and 1:96 respectively. The only previous New Zealand worker to test rat sera against *ballum* antigen was Brockie (1977) and he found 7 of 79 Norway rats (9%) and 1 of 16 ship rats (6%) to be seropositive at a minimum serum dilution of 1:100. The ratios of prevalence of Ballum serogroup titres to bacteriological prevalence in Norway and ship rats were very low (3:28 and 24:57 respectively) and this demonstrated the inadequacy of serology as a diagnostic method for the detection of Ballum serogroup infection in rats. The inability of organisms in this serogroup to stimulate high or sustained titres has been reported in several species (see Chapter IV and VII), however this inability was shown to be especially pronounced in the Norway rat. In the present study, 89% of isolations were from sero-negative animals and the GMT of sero-positive animals was very low.

Titres against antigens other than *ballum* were low and sporadic. It is probable that the *pyrogenes* titres represented cross-reactions in rats infected with leptospires of the Ballum serogroup as this cross-reaction is well recognised in a variety of species (Alston and Broom, 1958; Alexander *et al*,1963). The two low *tarassovi* titres in Norway rats may have represented infection with organisms of this serogroup, as serovar *tarassovi* has been isolated from Norway rats in East Europe (Anon, 1966).

The isolation of leptospires of the Ballum serogroup from Norway and ship rats in syanthropic foci supports the work by Brockie (1977) and two isolates were shown to be serovar *ballum*. Endemic Ballum serogroup infection was also found in ship rats in natural foci and therefore it can be concluded that infection with these organisms is widespread in rats in the southern half of the North Island of New Zealand.

Surveys of Norway rats in other countries, summarised in
Table 12.11, have revealed only low prevalences of Ballum serogroup
infection. In contrast, these surveys have revealed a high prevalence
of Icterohaemorrhagiae serogroup infection in the populations sampled.
The situation involving infection of Norway rats in New Zealand with
these two serogroups is summarised in Table 12.12. This comparative
data indicates that when endemic Icterohaemorrhagiae serogroup
infection occurs in a population of Norway rats, the recognised main-
tenance hosts, infection with organisms of the Ballum serogroup is
sporadic or absent. Analysis of data presented by Brockie (1977)
shows that no Ballum serogroup isolations were made from rats collected
from the Eastern Waikato, a population with endemic Icterohaemorrhagiae
serogroup infection. (*Copenhageni* infection has been documented in
cattle only in the South Auckland region (Chapter I) and it is probable
that Norway rats are the source of these infections.) The absence
of Icterohaemorrhagiae serogroup infection in the rat populations
studied in the present survey, together with the absence of other
serovars in rats in New Zealand, appears to allow a high prevalence
of Ballum serogroup infection to be maintained in some Norway rat
populations. This situation, the result of the particular ecology
of the Norway rat and the limited availability of other possible
infecting serovars in New Zealand, has not been reported in other
countries. The patchy distribution of the Norway rat in New Zealand
may mitigate against the spread of Icterohaemorrhagiae infection
in the southern half of the North Island, however it is suggested
that if these organisms were introduced, they would displace endemic
Ballum serogroup infection in Norway rat populations.

The present study has established that infection with leptospires
of the Ballum serogroup is endemic in ship rats in the southern half
of the North Island. Unlike the Norway rat, the ship rat is not a
recognised maintenance host for any particular serovar, although
many different isolations have been made from this species throughout
the world (Anon, 1966; Anon, 1975). These appear to be sporadic and
of local importance only and may be attributed to the very restricted
distribution of the ship rat in countries other than New Zealand.
Some surveys of ship rats have failed to reveal evidence of leptospiral
infection, even in environments where other free-living species are

Table 12.11 : Prevalence of Ballum and Icterohaemorrhagiae serogroup
infection in Norway rats.

| Author | Country | No. sampled | Ballum | | Icterohaemorrhagiae | |
|---|---|---|---|---|---|---|
| | | | No. | Prev. | No. | Prevalence |
| Brown & Gorman, 1960 | U.S.A. | 30 | 0 | - | 0 | - |
| Alexander *et al*, 1963 | Puerto Rico | 104 | 2 | 2% | 21 | 20% |
| Schnurrenberger *et al*,1970 | U.S.A. | 432 | 11 | 3% | 201 | 47% |
| Fennestad and Borg-Petersen,1972 | Denmark | 82 | 0 | - | 17 | 21% |
| Michna & Campbell, 1974 | U.K. | 134 | 1 | 1% | 20 | 15% |

Table 12.12 : Prevalence of Ballum and Icterohaemorrhagiae serogroup
infection in Norway rats in New Zealand.

| Author | No. sampled | Ballum infection | | Icteroharmorragiae | |
|---|---|---|---|---|---|
| | | No. | Prevalence | No. | Prevalence |
| Kirschner & Gray,1951 | 52 | 0 | - | 2 | 4% |
| Brockie, 1977 | 25* | 0 | - | 5 | 20% |
| | 51** | 8 | 16% | 0 | - |
| Hathaway, 1978 | 232 | 63 | 27% | 0 | - |
| | 123*** | 53 | 43% | 0 | - |

    * sample from Eastern Waikato

   ** sample from Southern Taranaki

 *** sample from Feilding tip.

recognised carriers (Clark *et al*,1966; Doherty, 1967; Fennestad and Borg-Petersen, 1972). The high prevalence of infection in ship rats in rural and forest biotopes indicates that this species is a maintenance host for Ballum serogroup organisms in New Zealand.

The prevalence of infection in Norway and ship rats in different biotopes provides some important information on the epidemiology of Ballum serogroup infection in these species. Norway rats from different rubbish tips in the Manawatu had prevalences of infection ranging from 3% to 43%, while those from suburban and farm locations were negative. The different rubbish tips sampled represented very similar biotopes and therefore the differences in prevalence of infection were independant of environmental conditions. Population densities on different tips were very different however, due to differences in tip management,and the prevalence of infection was directly associated with population density. High density populations of Norway rats were capable of maintaining a high prevalence of infection, whereas low density populations had either a low (Palmerston North tip) or zero prevalence of infection (Linton and suburban Palmerston North). These results indicate that a high frequency of contact is necessary to ensure transmission of the organism and hence maintenance of an endemic focus of Ballum serogroup infection in Norway rats. Such contact, both sexual and non-sexual, is maximal in crowded environments (Calhoun, 1962).

In comparison to Norway rats, the prevalence of infection in ship rats was independant of population density and was maintained at a high level in all but one of the populations sampled. Thus ship rat populations of moderate and low densities were capable of maintaining an endemic focus of Ballum serogroup infection, and the prevalence of infection was independant of different environmental conditions in different biotopes.

In contast to ship rats, the Norway rat may not be a typical maintenance host for leptospires of the Ballum serogroup and therefore a high frequency of contact between individuals in a population is needed to maintain the disease. The hypothesis that the Norway rat is not a typical maintenance host for leptospires of the Ballum serogroup is supported by several observations.

Plate 12.1. : High density population of Norway
rats at Feilding dump.

1.    The prevalence of infection has been shown to be density-dependant, with an absence of infection at low population densities.

2.    The Norway rat is a recognised host for *copenhageni*.  This serovar appears to be maintained independant of high population densities.

3.    *Copenhageni* appears to be maintained preferentially to *ballum*.

4.    Norway rats are a maintenance population of *ballum* only in syanthropic foci.

5.    Leptospires were isolated from very young Norway rats. This is atypical of infection in a maintenance host population of Norway rats (Babudieri, 1958; Kallai *et al*, 1962; Schnurrenberger *et al*, 1970).

6.    The GMT of *ballum* titres was very low (less than half the GMT for ship rats).  This is atypical of the serological criteria defining rodent species as maintenance hosts for leptospires (Chernukcha *et al*, 1974).

7.    Surveys of Norway rats in countries where these rodents share a common biotope with house mice with endemic *ballum* infection have shown that Norway rats are only sporadically infected or else remain uninfected in such biotopes.  (Brown and Gorman, 1960; Clark, 1961; Schnurrenberger *et al*, 1970). Thus the presence of *ballum* in wildlife in a particular biotope does not result in endemic infection in Norway rats.

Although the above-mentioned observations support the hypothesis that the Norway rat is not a typical maintenance host for leptospires of the Ballum serogroup, this hypothesis can only be tested by experimental investigations in the laboratory.

The absence of leptospiral infection in both Norway and ship

rats from suburban Palmerston North supports the findings of previous New Zealand workers who failed to find leptospirosis in rats in suburban and urban environments (Blakelock and Allen,1956; Brockie, 1977). The high level of predation by man and domestic animals in such environments ensures that rat populations are generally very low and this is the probable reason why endemic foci of Ballum serogroup infection are not maintained. (A similar situation was reported for a variety of serovars and rat species in urban compared with rural environments in Malaya by Gordon-Smith *et al*,1961). These findings indicate that, even in a maintenance host population, some minimum population density is necessary to maintain an endemic focus of infection of a particular serovar. This concept has been put forward by other workers investigating leptospirosis in free-living species (Brown and Gorman, 1960; Roth *et al*,1963) and Gordon-Smith *et al* (1961c)reported that in Malaya a minimum population density of two rats/ha was necessary to maintain leptospiral infection.

Ship rats from Linton were found to be infected with leptospires of the Ballum serogroup whereas Norway rats sharing the same biotope were negative. It is unlikely that different species sharing the same biotope have sufficient contact of the type that is necessary to transmit the disease between species. Gordon-Smith *et al* (1961c) investigating leptospiral infection in a variety of species of rats in several biotopes in Malaya, expressed a similar opinion. If any transmission does occur between species, it is probable that it is via the environment and as such is a rare occurrence.

The epidemiology of Ballum serogroup infection in Norway and ship rats is summarised schematically in Figure 12.4. The present investigation has demonstrated that the epidemiology of leptospirosis in rodent populations has multiple determinants and can only be defined in terms of local conditions. The maintenance of an endemic focus of leptospiral infection depends on continued transmission of the organism and this is only achieved under the following conditions:

1. suitable host
2. suitable host habitat
3. sufficient population density
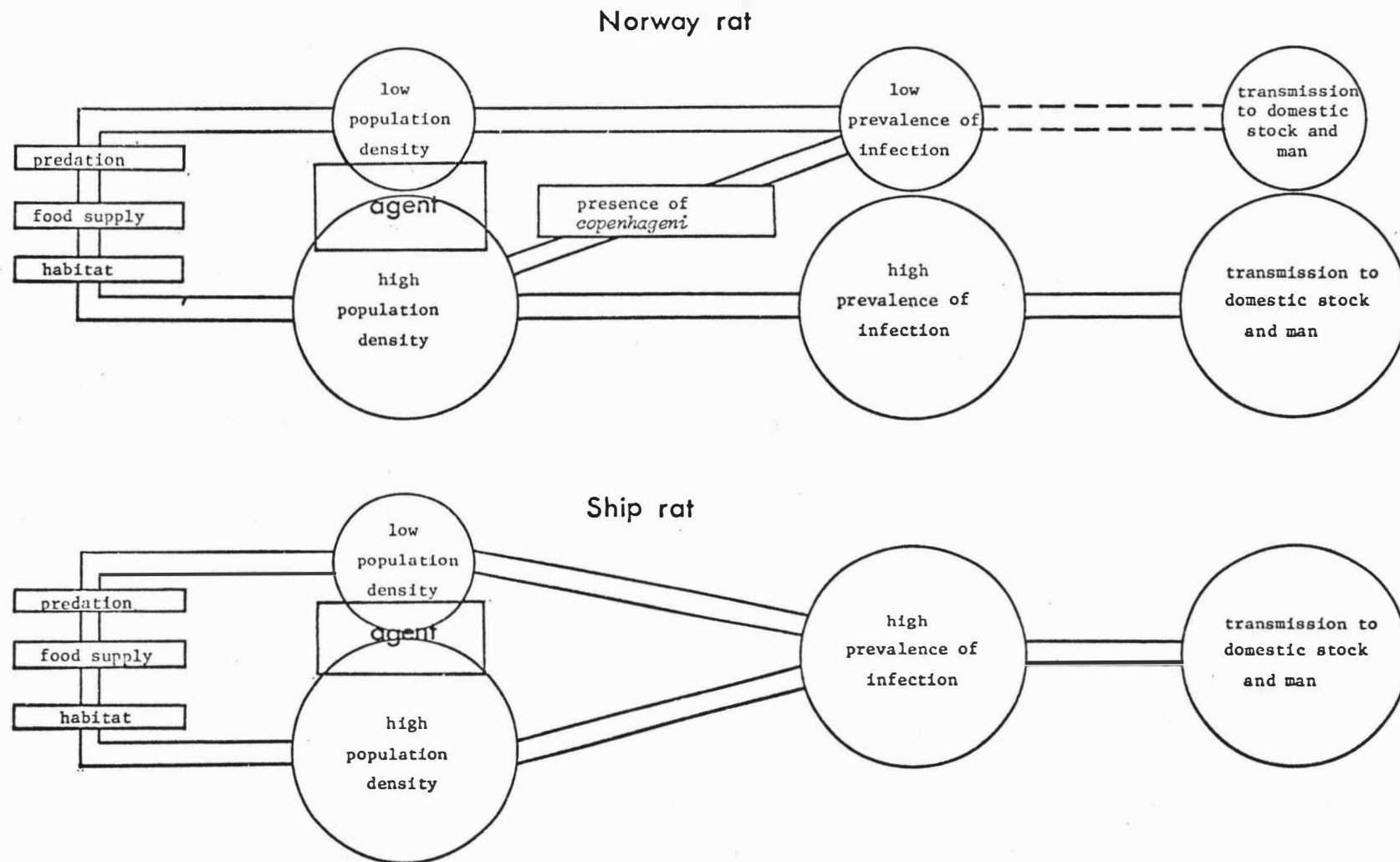4. introduction of a particular serovar.

**Figure 12.4. : Epidemiology of Ballum serogroup infection in the Norway and ship rat.**

A favourable environment for the survival of the infecting serovar is relatively unimportant for the maintenance of the focus under these conditions. This is in contrast to the opinions of many workers who have regarded a favourable environment to be important in the maintenance of an endemic focus of leptospirosis in a free-living population (Ferris *et al*, 1961; Gordon-Smith *et al*, 1961b; Turner, 1967; Twigg *et al*, 1969). A favourable environment is much more important for the transmission of the infectious agent to domestic animals and man. In most cases, the serovars that are endemic in free-living rodent populations are not endemic in domestic animals, due to the limitations of environmental transmission (Martin *et al*, 1967; Fennestad and Borg-Petersen, 1972; Twigg *et al*, 1972), however an increase in the population density of infected rodents in a biotope may result in an increase in the prevalence of sporadic infections in domestic animals and man sharing that biotope.

## SUMMARY AND CONCLUSIONS

1. Norway rats were only trapped in syanthropic foci, whereas ship rats were found to occupy both syanthropic and natural foci in New Zealand.

2. The prevalence of leptospiral titres in Norway and ship rats was very low. *Ballum* titres were found in 3% of Norway rats and 24% of ship rats.

3. Leptospires of the Ballum serogroup were isolated from 63 of 232 Norway rats (27%) and 17 of 52 ship rats (33%).

4. Isolations were restricted to the Ballum serogroup. Two isolates were identified by cross-agglutination absorption as serovar *ballum*. There was no evidence of infection with organisms of the Icterohaemorrhagiae serogroup.

5. Isolations were obtained from ship rats from rural and forest environments and the prevalence of infection was independant of population density.

6. The ship rat is considered a maintenance host for serovar *ballum* in the southern half of the North Island of New Zealand.

7. The prevalence of infection in Norway rats was directly related to population density.

8. Evidence is presented to support the hypothesis that the Norway rat is not a typical maintenance host for Ballum serogroup infection, however an endemic focus can be maintained in a syanthropic focus where there is a high population density.

9. Cross-species transfer of Ballum serogroup infection is considered unimportant in the maintenance of an endemic focus of leptospirosis in several species sharing a biotope.

10. The epidemiology of leptospiral infection in rats has multiple determinants and can only be defined in terms of local conditions.

CHAPTER XIII

## LEPTOSPIROSIS IN SMALL MAMMALIAN SPECIES, FERAL UNGULATES AND PUKEKOS (*PORPHYRIO MELANOTUS*) IN NEW ZEALAND.

### INTRODUCTION

Leptospiral infection occurs in a large number of free-living species throughout the world and it is well established that a particular free-living species may act as a maintenance host for different serovars in different countries. The European hedgehog is a maintenance host for *bratislava* in Great Britain (Michna and Campbell, 1970) and *canicola* in Israel (Shenberg *et al*, 1977), and the house mouse is a maintenance host for *ballum* in the U.S.A. (Clark, 1961) and *sejroe* and *bataviae* in Europe (Fennestad and Borg-Petersen, 1972; Mateev and Manev, 1974). In addition, a species with endemic leptospiral infection in one country may be free of infection in another. Surveys in Northern Ireland (McCaughey and Fairley, 1971) and Colarado, U.S.A. (Al Saadi and Post, 1976) have failed to demonstrate leptospiral infection in house mice in these regions.

New Zealand, unlike most countries, has a very limited number of free-living mammals and most of those that are established in this country were introduced from Great Britain in colonial times (see Chapter XI). This chapter describes an investigation of leptospirosis in these introduced species. Particular emphasis was placed on the collection of mustelids as no information is available on leptospirosis in these species in New Zealand and very few of these animals have been examined in other countries (Clark, 1961; Twigg *et al*, 1968; Michna and Campbell, 1970). The findings in free-living species described in this chapter provide an interesting comparison to the situation described for the same species in Great Britain.

### MATERIALS AND METHODS

The methods of collection for the different species have been described in Chapter XI. Animals were collected from a variety of locations and in general were obtained from those biotopes from which possums and rats were also sampled (Table 13.1). Mice were

trapped in the same forest ecosystems (Manawatu Gorge and Tiritea
Reserve) from which ship rats were trapped and a trapping programme
was also carried out in the same environment at Linton from which
Norway and ship rats were obtained. Two mice were trapped in subur-
ban Palmerston North.

Mustelids were trapped on White Rock and Otawhao farms
(described in Chapter IV) and also at Linton. Individual animals
were trapped at Dannevirke, Manawatu Gorge and Akatarawa Forests,
and Tangimoana. Individual feral cats were trapped on White Rock
and Otawhao farms and also at Dannevirke and Linton during trapping
programmes for other species. Six feral cats inhabiting the
Feilding tip were shot by spotlighting.

Hedgehogs were obtained from the grounds of Massey University,
Palmerston North tip and farmland around Linton. Individual hedgehogs
were captured on Otawhao farm and in suburban Palmerston North.

Several rabbits and hares were shot on farmland around
Dannevirke during night-shooting operations for possums. A sample
of 35 sera from feral ungulates and a feral pig, from various
localities in the North Island (Table 13.2), were subjected to
serological examination. Thirty-four pukekos collected from the
North and South Islands were also examined serologically.

The animals were weighed and sexed at the time of capture and
blood was taken for serology. Sera were tested in the MAT against
12 antigens, *viz., australis, autumnalis, ballum, bataviae, biflexa,
canicola, copenhageni, grippotyphosa, hardjo, pomona* and *pyrogenes,*
at a minimum serum dilution of 1:24.

Two methods were used for kidney culture, depending on the
species of animal. Kidneys of ferrets, stoats, feral cats,
hedgehogs, rabbits and hares were homogenised using the Stomacher
technique described in Chapter III. Kidneys from weasels and house
mice, because of their small size, were homogenised using the
syringe technique described in Chapter XII for ship rat kidneys.
Mouse kidneys were homogenised using a tuberculin syringe from which

the needle had been removed. Weasel kidneys were homogenised in a
2ml disposable syringe.

Culture of kidney homogenates and serogroup identification of
isolates were carried out according to the methods described in
Chapter III.

RESULTS

Trapping programmes for house mice resulted in only a small
number of animals being caught (Table 13.1). Five mice were trapped
in forest areas during 660 trap-nights, a trap-night average of 0.8
per 100 trap nights. A further five mice were trapped at Linton during
574 trap-nights, a trap-night average of 0.9 per 100 trap nights.

Mustelids also proved difficult to capture. Specific trapping
programmes for these species resulted in only eight animals being
captured, however a further 12 mustelids were captured during surveys
of other species. Capture of other small mammals was sporadic as these
species were not subjected to intensive trapping programmes.

Serological and cultural results are presented in Table 13.3.
No leptospiral agglutinins were detected in sera from house mice,
however two isolates of the Ballum serogroup were obtained from this
species. Both isolates were from adult mice, one from Manawatu
Gorge Forest and one from Linton. The overall bacteriological
prevalence in mice was 17%.

The nine ferrets, eight stoats and three weasels that were
captured showed no serological evidence of leptospiral infection and
no isolates were obtained from these species.

Two of 11 feral cats were found to have serological evidence
of leptospiral infection. One adult male from White Rock Farm had a
titre of 1:96 to *pomona* and an adult female from Otawhao farm had a
titre of 1:24 to *ballum*. No isolates were obtained from feral cats.

*Ballum* titres were found in 4 of 13 hedgehog sera (31%) and the

Table 13.1 : Collection data for small mammalian species and birds
investigated for leptospiral infection.

| Order | Species | Date | Location | Sex | Weight (g) |
|---|---|---|---|---|---|
| RODENTIA | House Mouse (*Mus musculus*) | 10.7.76 | Manawatu Gorge | M | 19 |
| | | 15.6.76 | " | M | 12 |
| | | 15.9.76 | Tiritea Reserve | F | 16 |
| | | 28.9.76 | " | F | 14 |
| | | " | " | M | 17 |
| | | 12.4.77 | Linton | F | 17 |
| | | 30.9.77 to 16.10.77 | Linton | M | 12 |
| | | | " | F | 20 |
| | | | " | M | 9 |
| | | 13.11.77 | " | M | 12 |
| | | 25.6.77 | Palmerston Nth City | M | 15 |
| | | 20.11.77 | " | M | 23 |
| | Kiore (*Rattus exulans*) | 30.11.76 | Arapawa Island | M | NT |
| | | " | " | M | NT |
| CARNIVORA | Ferret (*Putorious putorious*) | 10.3.76 to 16.3.76 | White Rock Farm | F | NT |
| | | | " | F | NT |
| | | " | " | M | NT |
| | | " | " | M | NT |
| | | " | " | M | NT |
| | | 10.8.76 | Otawhao Farm | M | NT |
| | | 18.5.77 | " | F | NT |
| | | 23.6.77 | " | F | NT |
| | | 14.8.77 | Linton | M | NT |
| | Stoat (*Mustela erminea*) | 10.8.75 | White Rock Farm | F | NT |
| | | 13.3.76 | " | F | NT |
| | | 16.3.76 | " | M | NT |
| | | 10.4.76 | Dannevirke | F | NT |
| | | 3.6.76 | Manawatu Gorge | M | NT |
| | | 10.8.76 | Otawhao Farm | M | NT |
| | | " | " | M | NT |
| | | 18.10.77 | Linton | M | NT |

Table 13.1 cont.

| Order | Species | Date | Location | Sex | Weight (g) |
|---|---|---|---|---|---|
| CARNIVORA (cont.) | Weasel *(Mustela nivalis)* | 16.12.76 | Tangimoana | M | 140 |
| | | " | " | F | 165 |
| | | 15.5.77 | Akatarawa Forest | F | 190 |
| | Feral cat *(Felis catus)* | 11.3.76 | White Rock Farm | M | NT |
| | | 15.3.76 | " | F | NT |
| | | 19.5.77 | Otawhao Farm | F | NT |
| | | 20.7.77 | Dannevirke | F | 1600 |
| | | 25.10.77 | Feilding tip | M | 1210 |
| | | " | " | F | 2950 |
| | | 27.10.77 | " | M | 1575 |
| | | " | " | N | 1240 |
| | | " | " | F | 1140 |
| | | " | " | F | NT |
| | | 2.3.78 | Linton | M | 1875 |
| INSECTIVORA | Hedgehog *(Erinaceus europaeus)* | 9.10.76 | Massey University | M | 310 |
| | | 15.10.76 | " | M | 375 |
| | | 3.3.77 | " | F | 525 |
| | | " | " | F | 430 |
| | | 15.5.77 | Palmerston Nth tip | M | 510 |
| | | 7.10.77 | " | F | 635 |
| | | " | " | M | 470 |
| | | " | " | M | 400 |
| | | " | " | M | 545 |
| | | 28.6.77 | Otawhao Farm | M | 495 |
| | | 13.2.78 | Linton | F | 825 |
| | | " | " | F | 210 |
| | | " | " | M | 540 |
| | | 11.10.77 | Palmerston Nth City | M | 325 |
| LAGOMORPHA | Rabbit *(Oryctolagus cuniculus)* | 29.3.77 | Dannevirke | M | NT |
| | | " | " | M | NT |
| | | 28.10.77 | " | M | NT |
| | | " | " | F | NT |
| | | " | " | F | NT |
| | | " | " | M | NT |
| | | " | " | F | NT |

Table 13.1 cont.

| Order | Species | Date | Location | Sex | Weight (g) |
|---|---|---|---|---|---|
| LAGOMORPHA | Hare *(Lepus europaeus)* | 28.10.77 " | Dannevirke " | M M | NT " |
| | * Pukeko *(Porphyrio melanotus)* | – | various | mixed | NT |

* 34 serum samples only

Table 13.2 : Species of wild ungulates examined for serological evidence of leptospiral infection.

| Species | Date of Collection | Location | Sex |
|---|---|---|---|
| *Red deer *(Cervus elaphus)* | May,1977 | North Island | Mixed |
| Red deer | 17.8.77 | Putorino | F |
| Red deer | 20.8.77 | " | M |
| Sika deer *(Cervus nippon)* | 14.5.77 | Taupo | M |
| Feral sheep *(Ovis aries)* | November,1976 | Arapawa Is. | M |
| Feral sheep | " | " | M |
| Feral sheep | " | " | M |
| Feral sheep | " | " | F |
| Feral sheep | " | " | F |
| Feral sheep | " | " | F |
| Feral goat *(Capra hircus)* | " | " | NT |
| Feral goat | " | " | NT |
| Feral pig *(Sus scrofa)* | 14.3.77 | Putorino | M |

* pooled sample of 25 red deer sera from various North Island locations.

range in titres was from 1:24 to 1:384, with a GMT of 1.68.
Multiple reactions against a variety of heterologous antigens were
found in the sera of two hedgehogs (Table 13.3). Isolates of the
Ballum serogroup were cultured from both of these animals.

Rabbits and hares were serologically and culturally negative.
Serum from the two kiore were also negative and no titres were
detected in sera from 36 feral ungulates and a feral pig.

No titres were demonstrated in the 34 pukeko sera that were
examined.

## DISCUSSION

The isolation of leptospires of the Ballum serogroup from house
mice, ship rats, Norway rats and hedgehogs and the serological evid-
ence of infection in possums in New Zealand is further evidence of
the ability of these organisms to infect a wide range of free-living
species throughout the world. Unlike most other countries, *ballum*
is one of only two serovars that have been isolated from rodents
and hedgehogs in New Zealand. Leptospires of the Ballum serogroup
have also been isolated from cattle and humans in this country
(see Chapter I).

The house mouse is recognised as a maintenance for *ballum* in
many regions throughout the world,(van der Hoeden, 1958; Brown and
Gorman, 1960; Martin *et al*, 1967). Brockie (1977) trapped 73 mice
from dairy farms and country rubbish tips in the North Island of
New Zealand and isolated leptospires of the Ballum serogroup from
nine (12%), so establishing the presence of these organisms in
house mice in this country. Only two mice in that sample had *ballum*
titres at a minimum serum dilution of 1:200. These results were
similar to those found in the present investigation and so the house
mouse can be considered a maintenance host for leptospires of the
Ballum serogroup in New Zealand. (This conclusion is supported by
the results from the culture of 58 mice described in Chapter XV).
The isolation of leptospires from a mouse in a natural focus
(Manawatu Gorge Forest) demonstrates that Ballum serogroup infection
in house mice is not restricted to syanthropic foci.

Table 13.3 : Serological and cultural evidence of leptospiral infection in small mammalian species.

| Species | Location | Sex | Weight(g) | Titre | | Culture | Isolate serogroup |
|---------|----------|-----|-----------|-------|---|---------|-------------------|
| House mouse | Manawatu Gorge | M | 19 | − | | + | Ballum |
| | Linton | F | 17 | − | | + | Ballum |
| Feral cat | White Rock Farm | M | NT | 1:96 | *pomona* | − | |
| | Otawhao Farm | F | NT | 1:24 | *ballum* | − | |
| Hedgehog | Palmerston North tip | M | 545 | 1:384 | *ballum* | + | Ballum |
| | | | | 1:48 | *copenhageni* | | |
| | | | | 1:48 | *canicola* | | |
| | | | | 1.24 | *pomona* | | |
| | Otawhao Farm | M | 495 | 1:96 | *ballum* | + | Ballum |
| | | | | 1:24 | *copenhageni* | | |
| | | | | 1:24 | *pyrogenes* | | |
| | Massey University | F | 525 | 1:24 | *ballum* | − | |
| | Linton | F | 825 | 1:24 | *ballum* | − | |

Table 13.4 : Summary data for leptospiral infection in small mammalian species.

| Species | No.collected | Sex | | No. serum samples | No. positive sera | Isolations* | | |
|---|---|---|---|---|---|---|---|---|
| | | M | F | | | M | F | Total |
| House mouse | 12 | 8 | 4 | 4 | 0 | 1 | 1 | 2 |
| Kiore | 2 | 2 | 0 | 2 | 0 | NT | NT | NT |
| Ferret | 9 | 5 | 4 | 9 | 0 | 0 | 0 | 0 |
| Stoat | 8 | 5 | 3 | 8 | 0 | 0 | 0 | 0 |
| Weasel | 3 | 1 | 2 | 3 | 0 | 0 | 0 | 0 |
| Feral cat | 11 | 5 | 6 | 11 | 2 | 0 | 0 | 0 |
| Hedgehog | 14 | 9 | 5 | 13 | 4** | 2 | 0 | 2 |
| Rabbit | 7 | 4 | 3 | 6 | 0 | 0 | 0 | 0 |
| Hare | 2 | 2 | 0 | 2 | 0 | 0 | 0 | 0 |
| Total | 68 | | | 58 | 6 | 3 | 1 | 4 |

* all isolations were of the Ballum serogroup

** two sera had titres to more than one antigen.

The house mouse does not appear to be a maintenance host for *ballum* in Great Britain, although only small numbers have been investigated (Twigg *et al* 1968; Michna and Ellis, 1974).( Twigg *et al* (1972) refer to "the well-known association of *L.ballum* with the house mice" but do not state whether they are referring to the situation in countries other than Britain or the the local situation ). The house mouse is a maintenance host for *sejroe* in Europe (Fennestad and Borg-Petersen, 1972). These authors isolated *sejroe* from 25 of 220 house mice (11%). Only one *ballum* isolate was obtained in that survey. Parnas *et al* (1961) cultured 17 isolates from 1090 house mice, none of which were *ballum*. Thus the epidemiology of leptospirosis in house mice in New Zealand is considerably different from that in Great Britain and Europe.

The 20 mustelids that were examined revealed no evidence of leptospiral infection. This is an interesting finding in view of the fact that an important part of the diet of these carnivores in New Zealand consist of rodents (see Chapter XI). Despite the presence of endemic Ballum serogroup infection in ship rats, house mice, and some high density populations of Norway rats, there appears to be no predator-chain transmission of this infection to mustelids. Predator-chain transmission has been demonstrated experimentally from *ballum*-infected mice to hedgehogs (van der Hoeden, 1964) and evidence of infection by this route was reported by Reilly (1970), who fed *grippotyphosa*-infected mice to skunks and red foxes. Other workers have failed to demonstrate experimental predator-chain transmission (Kiktenko *et al*,1976). Although several workers have postulated that predator-chain transmission occurs in natural foci of leptospirosis (Catchpole,1934; Medinskii,1959; Schnurrenberger *et al*, 1970),there is little field evidence available supporting this hypothesis.

Relatively few mustelids have been investigated in other countries. Michna and Campbell (1970) found one of eight weasels in Great Britain serologically positive to *sejroe* and Twigg *et al* (1968) found a titre of 1:100 to *bratislava* in one of three weasels. No isolations were made by these workers. Fennestad and Borg-Petersen (1972) cultured serovars *poi, pomona* and *sejroe* from 3 of 37 stoats, however 11 polecats

*(Mustela putorius)* and 16 weasels were negative. Two weasels
cultured in the U.S.A. by Clark (1961) were also negative whereas
house mice sharing the same biotope as the weasels had endemic
*ballum* infection. It therefore appears that mustelids are not
significant carriers of leptospirosis.

Feral cats have not been previously investigated in New
Zealand. No isolates were obtained from this species in the
present survey, however serological evidence of infection was
found in two cats. One feral cat had a titre of 1:96 to *pomona*,
a serovar that has been isolated from a domestic cat in New
Zealand (Harkness *et al*,1970). The epidemiological significance
of these *pomona* infections for domestic stock is unknown, however
it should be noted that evidence of *pomona* infection has not been
found in any other small free-living species in New Zealand.
Andrews and Ferris (1966) isolated *pomona* from a feral cat in the
U.S.A.

The titre of 1:24 to *ballum* in a feral cat from Otawhao Farm
may have been due to predator-chain transmission from endemically-
infected rodents on this property, but the absence of *ballum* titres
in the six feral cats from Feilding tip mitigates against this
possibility. Five of the six cats from Feilding tip had rat tissue
and fur in their stomach contents and therefore, despite the high
level of predation on a Norway rat population with endemic Ballum
serogroup infection, there was no evidence of transmission of the
disease to cats. Clark (1961) also failed to isolate leptospires
from 56 cats in Pennsylvania, U.S.A., even though endemic *ballum*
infection occurred in house mice in the same region.

Another free-living species shown to be infected with lepto-
spires of the Ballum serogroup was the European hedgehog, with
isolations being obtained from one adult from Otawhao Farm and
an adult from Palmerston North rubbish tip. Brockie and Till (1977)
isolated organisms of the Ballum serogroup from 5 of 72 hedgehogs
from dairy farms in the North Island (7%) and four isolates were
typed by cross-agglutination absorption as being serovar *ballum*.
Only females in that sample were bacteriologically positive,however
both isolates in the present survey were from males.

These results demonstrate that the hedgehog is another free-living species in New Zealand with endemic Ballum serogroup infection. The ratios of serological to bacteriological prevalence in the study by Brockie and Till (1977) and the present study were 28:5 and 2:1 respectively and therefore, according to the criteria of Roth *et al* (1963), the hedgehog appears to be a less efficient maintenance host for Ballum serogroup infection than the house mouse and ship rat.

A considerable number of heterologous titres were found in the sera from hedgehogs with Ballum serogroup infection and it is considered that these were due to cross reactions rather than representative of multiple infections. Brockie and Till (1977) reported an even higher degree of cross-reactivity in hedgehog sera with positive *ballum* titres. This cross-reactivity included heterologous titres to *pomona* antigen.

Webster (1957) claimed that hedgehogs may be important in the epidemiology of *pomona* infection in bovines in New Zealand. *Pomona* titres were reported in two hedgehogs found on a dairy farm on which there had been an outbreak of *pomona* infection in cattle and one of these hedgehogs had a leptospiruria. No attempts were made at isolation of the infecting leptospires and the screening of sera against antigens other than *pomona* was not attempted. This worker experimentally infected juvenile and adult hedgehogs with *pomona* and produced a fatal infection in the young animals and severe clinical disease in adults. Severe manifestations of disease are atypical of leptospiral infection in a maintenance host and this indicates that it was unlikely that *pomona* was the cause of the natural infections. Therefore the assumption that Webster (1957) was dealing with endemic *pomona* infection in hedgehogs on dairy farms is open to question.

The hedgehog in Great Britain and Europe is a recognised maintenance host for *bratislava*, with a range in bacteriological prevalence of from 8% to 26% having been reported (McDiarmid, 1965; Wolff and Bohlander, 1965; Michna, 1970). A summary by Wolff and Bohlander (1965) detailing the leptospiral serovars isolated from hedgehogs in Europe showed that *ballum* isolations were very rare, a contrary situation to that occurring in New Zealand.

The rabbits and hares examined in the present survey were serologically and culturally negative. The numbers examined were small and therefore the potential ability of Lagomorpha to carry leptospires in New Zealand remains in question. Blackmore *et al* (1976) isolated an organism belonging to the Ballum serogroup from 1 of 17 rabbits collected near Palmerston North, further illustrating the ubiquitous nature of these organisms in New Zealand wildlife. Five hares collected from the same locality were serologically and culturally negative. Serological surveys of Lagomorpha by workers in other countries have demonstrated titres against several antigens, including *bratislava, bataviae* and *sejroe* (McDiarmid, 1965; Twigg *et al*, 1968; Fennestad and Borg-Petersen, 1972). The significance of these titres remains largely undetermined.

Ballum serogroup infection described in house mice, hedgehogs and ship rats (Chapter XII) in New Zealand has similar characteristics in all species. Isolations were made only from adult animals and this is characteristic of endemic infection in maintenance host populations. The *ballum* titres recorded in each species were very low (the maximum titre was 1:384) and this supports the suggestion put forward in earlier chapters that Ballum serogroup leptospires have low antigenicity for free-living species. Serological and bacteriological evidence of infection was not present in animals from suburban environments except hedgehogs and similar findings were reported by Smith (1964) and Brockie and Till (1977). Hedgehogs are present in relatively high numbers in suburban environments and therefore population density would not be a limiting factor in the maintenance of endemic infection. The contrary situation occurs in rodents in suburban environments, as these animals are subjected to heavy predation by domestic animals and man, and therefore lack of endemic Ballum serogroup infection in these species may be due to discontinuous, low-density populations.

It is probable that interspecies transmission is not necessary to maintain Ballum serogroup infection in the free-living species found to be carriers. The prevalence of infection in different species ranged from 14% in hedgehogs to 50% in ship rats (Chapter XII) and this indicates that endemic infection was present in each species. The

ubiquitous presence of these organisms in wildlife in New Zealand is not found to the same extent in other countries where Ballum serogroup infection occurs in free-living populations. Workers in the U.S.A. have reported a similar prevalence of infection in the house mouse, a recognised maintenance host of *ballum* in many countries, however the prevalence of *ballum* infection in other free-living species sharing the same ecosystem was less than 3% (Brown and Gorman, 1960; Clark, 1961; Schnurrenberger *et al*, 1970). This may have been due to the presence of endemic infection with other serovars in species other than the house mouse, which were preferentially maintained compared with *ballum*, and thus prevented more than only sporadic *ballum* infection. The reason for the relatively high prevalence of *ballum* in hedgehogs, ship rats and some Norway rat populations in New Zealand may be due to the absence of leptospires from other serogroups (except *copenhageni* in Norway rats in isolated foci). This concept of "competitive exclusion" of a serovar from a particular free-living species in an ecosystem where several serovars are present has not been advanced by other workers, although cross-immunity betweeen some serovars has been experimentally demonstrated (Kemenes, 1964; Plesko, 1974).

The absence of titres in sera from feral ungulates from various localities supports the work of Daniel (1966; 1967), which is summarised in Chapter I. It is probable that feral ungulates are unimportant in the epidemiology of leptospirosis in domestic animals and man in New Zealand. Extensive serological surveys of deer in the U.S.A. have shown high prevalences of *pomona* and *grippotyphosa* titres (Trainer *et al*, 1963; Andrews *et al*, 1964: Shotts and Hayes, 1970) however the few cultural surveys that have been attempted have revealed only very low bacteriological prevalences of infection (Abdulla *et al*, 1962; Reilly *et al*, 1962). Thus deer appear to be only accidental hosts for leptospires. Very few titres have been reported in serological surveys of deer in Great Britain (Twigg *et al*, 1973) and Tasmania (Munday, 1972)

Pukekos are one of the most common wading birds on farmland in New Zealand. The absence of titres in these species is further evidence of the insusceptibility of birds to leptospiral infection (Torten *et al*, 1965).

## SUMMARY AND CONCLUSIONS

1.  Leptospires of the Ballum serogroup were isolated from house mice in natural and syanthropic foci.  The house mouse is considered to be a maintenance host for organisms of the Ballum serogroup in New Zealand.

2.  Mustelids were serologically and culturally negative and therefore there was no evidence of predator-chain transmission of leptospires from infected rodents to these species.

3.  Serological evidence of *pomona* and *ballum* infection was found in two feral cats.

4.  Ballum serogroup leptospires were isolated from hedgehogs and these animals are also considered maintenance hosts for leptospires of the Ballum serogroup in New Zealand.

5.  The concept of "competitive exclusion" is put forward as an explanation for infection with different serovars in the same species in different ecosystems.

6.  Rabbits and hares were serologically and culturally negative.

7.  No serological evidence of infection was found in feral ungulates and these species are considered unimportant in the epidemiology of leptospirosis in domestic animals and man in New Zealand.

8.  Thirty-four pukekos were serologically negative.

CHAPTER XIV

THE NIDALITY OF LEPTOSPIROSIS IN AN INTENSIVE FARMING
ENVIRONMENT

INTRODUCTION

The prevalence of leptospiral infection in various free-
living domestic animal and human populations in New Zealand has
been studied in a variety of surveys but there have been very few
investigations to determine the specific factors affecting intra-
and interspecies transmission in a particular environment.
Epidemiological studies at an artificial breeding centre and a
commercial piggery (Blackmore et al,1976; Buddle and Hodges,1977)
have described some of the factors affecting intraspecies trans-
mission of particular serovars, however,there has been little
comprehensive effort made to determine the importance of interspecies
transmission in such nidi in New Zealand.

Circumstantial evidence of interspecies transmission between
wildlife and domestic animals of a variety of serovars has been
reported in several investigations in other countries (Clark,1961;
Martin et al,1967; Twigg et al,1969; Twigg et al,1972). Very little
can be extrapolated from these investigations to the situation exist-
ing in New Zealand. There has been no long-term evolution of lepto-
spiral serovars in natural ecological niches in New Zealand and it
has been shown in previous chapters that the pattern of leptospirosis
in wildlife is very different to that occurring in other countries.

The presence of a commercial piggery and an adjacent town supply
dairy farm at Massey University provided an ideal opportunity to
study interspecies transfer of leptospirosis between wildlife and
domestic animals in a restricted environment over an extended period
of time. The investigation of wildlife inhabiting a syanthropic
focus that is described in this chapter was part of a collaborative
study involving wildlife, cattle (Hellstrom,1978) and pigs (Ryan,
1978). The dynamics of leptospiral infection in each species was
monitored over a period of two years.

MATERIALS AND METHODS

Description of Massey Pig Research Centre and Number 1 Dairy Farm.

The Massey Pig Research Centre is an intensive commercial pig unit managing approximately 600 pigs. The herd is made up of two groups; the growers (birth to eight months) and the breeding herd (sows and boars). The grower population is maintained by piglets from the breeding herd, whereas replacement gilts and boars for the breeding herd are bought in from other farms. Approximately 20% of the sow herd is replaced annually. Pigs in the breeding herd are occasionally grazed on paddocks surrounding the piggery buildings while growers are maintained indoors at all times. Replacement gilts are also grazed on paddocks for limited periods.

The Massey No. 1 Dairy Farm milks a town supply herd of approximately 130 cows. The cows calve in autumn and spring and replacement calves for the milking herd are raised on the property. The two batches of calves that are raised each year are meal-fed in a calf battery adjacent to the milking shed. The herd is maintained virtually as a closed unit by the use of an artificial breeding programme.

The layout of the piggery and the dairy farm is shown in Figure 14.1. The pastureland is generally flat and well-drained although considerable surface water is present during periods of winter rain. Buildings, shelter belts, plantations and an extensive area of trees and grass alongside the Manawatu River provide shelter for free-living species.

A potential opportunity for interspecies transfer of leptospirosis between cattle and pigs occurred when these animals were grazed in adjacent paddocks and also when the milking herd was moved along the raceway on the boundary of the piggery. In addition, surface water from the environs of the piggery drained into a large depression containing an eastward-flowing creek which bisects the dairy farm.

Figure 14.1. : Massey Pig Research Centre and No.1 Dairy Farm showing trapping and environmental sampling locations.
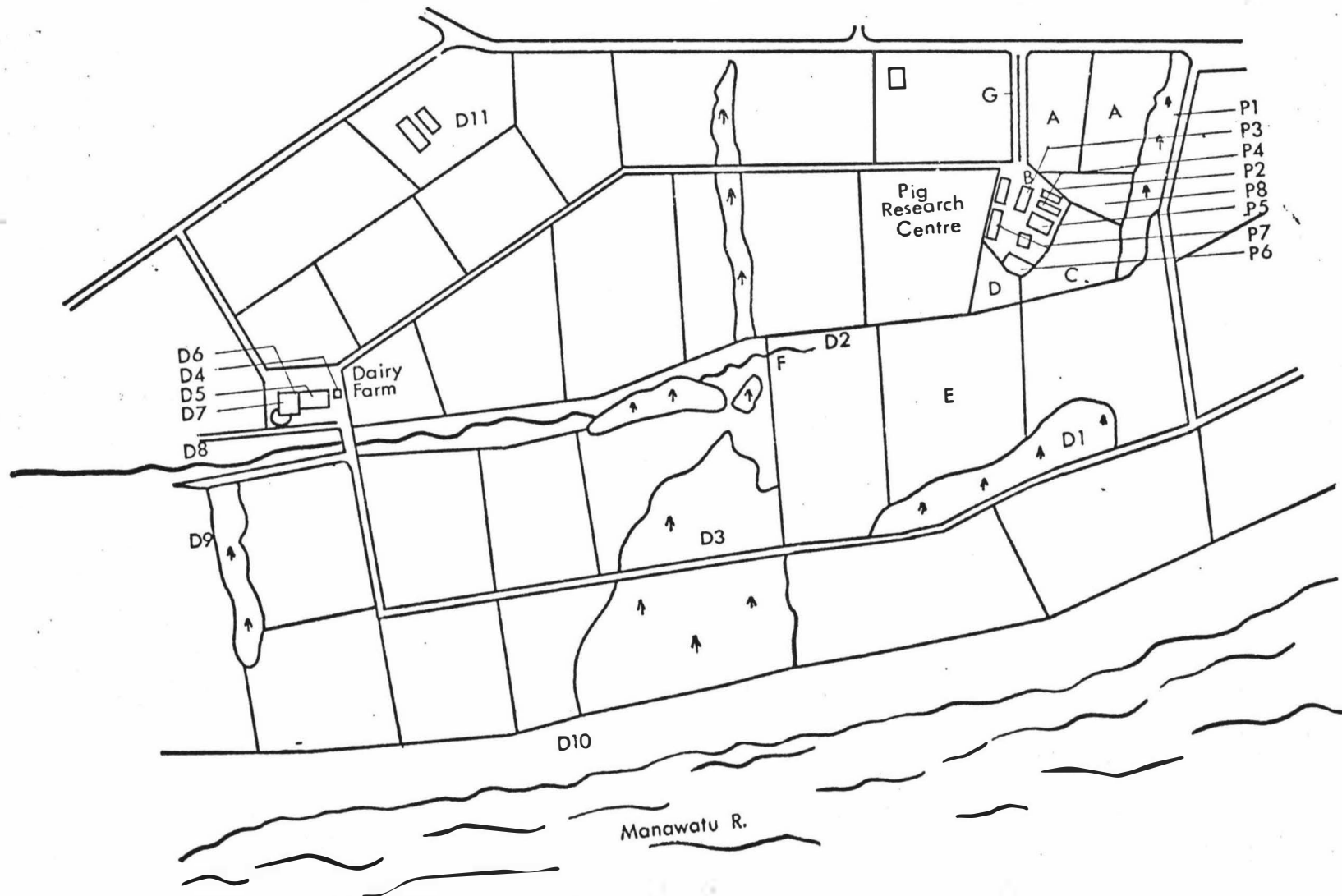
Figure 14.1 : Key to trapping and environmental sampling locations

Piggery

P1.  Bluegum and macrocarpa shelter belt. Extensive ground cover of long grass, shrubs and debris.

P2.  Effluent drain from piggery.

P3.  Grower house.

P4.  Weaner house.

P5.  Farrowing house.

P6.  Sow and boar pens.

P7.  Meal store.

P8.  Poorly drained grass paddock with standing surface water.

No. 1 Dairy Farm

D1.  Pine shelter belt. Well drained and very little ground cover.

D2.  Poorly drained, swampy depression surrounded by willows. Large pond forms here in winter.

D3.  Pine plantation. Well drained and very little ground cover.

D4.  Sawdust dump.

D5.  Calf-rearing battery.

D6.  Grain store.

D7.  Milking shed yards.

D8.  Creek enclosed by embankments. Long grass cover.

D9.  Macrocarpa shelter belt. Considerable ground debris.

D10. River bank. Extensive area of long grass, willows and poplars.

D11. Silage pit.

Environmental sampling locations

A    Gilt grazing paddocks.

B    Piggery effluent drains.

C    Sow and gilt grazing paddock containing stagnant pond.

D    Sow and gilt grazing paddock.

E    Flat dairy farm paddock.

F    Standing surface water in poorly drained dairy farm paddock.

G    Raceway used by milking cows.

Investigation of Leptospiral Infection in Wildlife

The trapping locations for wildlife are shown in Figure 14.1.
A variety of methods were used and these are described in general
terms in Chapter XI. Eighteen cage traps and 16 snap traps were
utilized for the capture of rats and 6 cage-traps ("Katch-All") and
18 snap traps were utilized for the capture of mice. Possums were
captured by the use of 14 steel traps. Hedgehogs were collected
by spotlighting on open pasture and around farm buildings at night.
Spotlighting was also used in an attempt to shoot rabbits which
inhabited the river bank which constituted the northern boundary
of the No. 1 Dairy Farm.

Wild grey ducks (*Anas superciliosa*) and mallard ducks *(Anas
platyrynchos)* were frequently observed in and around the piggery
from May until December each year. They fed on pig meal that was
washed into effluent drains during hosing down operations, and
also in a stagnant pond located in a sow paddock at the rear of the
piggery. It was considered that this feeding behaviour would
subject the ducks to a maximal challenge from leptospires shed by
penned and grazing pigs and therefore this species was also
investigated for evidence of infection.

Ducks were captured in a large, two-chambered, wire-mesh
trap (Plate 14.1). One-way funnels leading into each chamber
prevented trapped  ducks from escaping and they were removed from
the trap via folding roof sections. The trap was pre-baited with
pig meal and wheat without the funnels in place for eight days
before setting.

Free-living animals were captured during four main trapping
periods of four weeks each which took place in June and July 1976,
January and February 1977, June 1977 and November and December 1977.
Traplines were checked daily. Initial attempts to trap ducks in May
1976 resulted in only a small number being trapped and the majority
of ducks were trapped in December 1976.

Particular attention was paid to the trapping of wildlife that
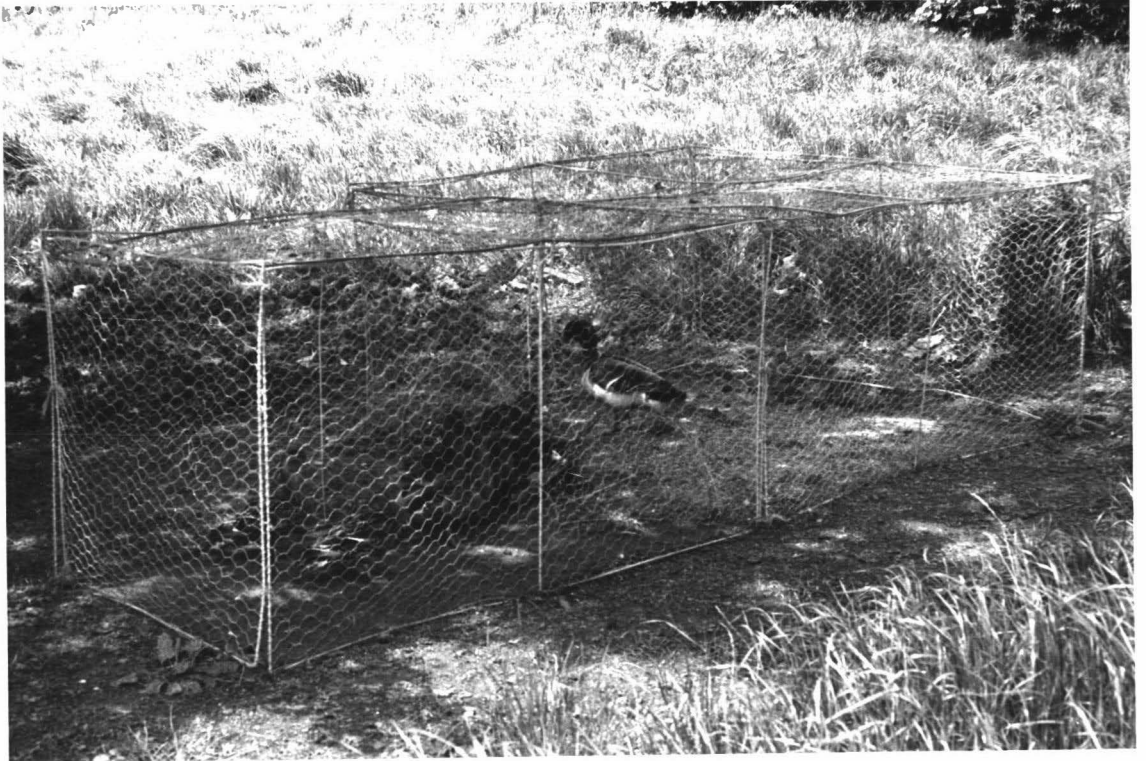inhabited areas where there was a maximum opportunity for either

Plate 14.1. : Double-chambered duck trap in use
at the Massey Pig Research Centre.

direct or indirect transmission of leptospirosis to and from domestic stock. Thus the majority of the trapping effort was focused on the piggery buildings, calf battery, milking shed and around effluent drains and areas of standing water. The number of trap-nights at each location during different trapping periods are given in Table 14.1.

The serological and cultural techniques used for examination of each species of free-living animal have been described in previous chapters. All sera were tested against *ballum*, *copenhageni*, *hardjo*, *pomona* and *tarassovi* antigens at a minimum serum dilution of 1:24. Possum sera were also tested against *balcanica* antigen.

Ducks were anaesthetised with ether and bled by severing the blood vessels in the neck. Both kidney and liver from ducks were cultured. Approximately one gram portions of these organs were aseptically removed and homogenised by forcing through a five-ml, sterile disposable syringe into nine mls. of Stuart's base medium. Dilution of the primary homogenate and inoculation of media was carried out as described in Chapter III for the Stomacher technique.

Attempts to trap mice in snap traps during the first trapping period in June and July,1977 met with little success and because mice were killed when trapped, no sera was available for serology. Imported "Katch-All" traps were introduced in January 1977 and the efficiency of these traps compared with snap traps is reflected in the trap-night averages achieved after their introduction.

Analysis of cultural data required that mice, ship rats and hedgehogs be differentiated into juvenile and sexually-mature age-groups. Ship rats weighing less than 82 g. were classed as juveniles (see Chapter XI). In the absence of scientific data on the weight of house mice at the onset of sexual maturity (see Chapter XI), immature mice were subjectively assessed to be those weighing less than 13 g. There is a similar lack of data on the onset of sexual maturity in the hedgehog under New Zealand conditions and therefore the criterion used to differentiate juvenile and sexually-mature hedgehogs was that used by Wolff and Bohlander (1965) in Holland. These workers classed hedgehogs weighing less than 500 g. as immature.

Investigation of leptospiral infection in domestic animals

Leptospiral infection in pigs was monitored by the testing of random samples of sera obtained every four months from the grower and breeding populations. In addition, selected groups of sows, boars and gilts were subjected to serological examination and urine samples were cultured for leptospires. Representative samples of serum and urine were also obtained from bought-in gilts when they arrived on the property.

A total of 700 sera obtained by the serial bleeding of 131 cows over the duration of the study were tested for leptospiral agglutinins. Representative samples of spring and autumn calves were serially bled at three month intervals for 18 months to determine the age at which dairy cattle first became infected. Serial urine samples from these animals were cultured and also monitored by darkfield microscopy.

Environmental sampling

Thirty environmental samples of piggery effluent, soil and surface water from paddocks grazed by pigs and cattle were processed for the isolation of leptospires. The locations from which environmental samples were taken are given in Figure 14.1. Soil samples were suspended in physiological saline and one ml of supernatant was inoculated intra-peritoneally into each of two weanling hamsters. Effluent and surface water samples were inoculated directly into hamsters and also subjected to the floating membrane technique of Fowler (1970) to remove contaminants before culturing. The culture of kidneys from hamsters has been described previously. All hamsters still alive three weeks post-inoculation were killed and also cultured.

RESULTS

Wildlife

The results of the trapping programme for wildlife are given in Tables 14.1 and 14.2. A total of 58 house mice, 13 Norway rats, 11 ship rats, 13 hedgehogs, 8 possums, 2 feral cats, 2 rabbits,

Table 14.1 : Trapping data for rodents from the Massey Pig Research
Centre and No. 1. Dairy Farm.

| Date | Species | Trap location | Trap-nights | No.caught | Trap-night average |
|------|---------|---------------|-------------|-----------|--------------------|
| June/ July, 1976 | Mice | P1 | 105 | 1 | 1:105 |
| | | P2 | 28 | 0 | 0 |
| | | P3 | 100 | 2 | 1:50 |
| | | P4 | 84 | 1 | 1:84 |
| | | P5 | 84 | 0 | 0 |
| | | P6 | 42 | 0 | 0 |
| | | P7 | 84 | 0 | 0 |
| | | D1 | 28 | 0 | 0 |
| | | D3 | 28 | 0 | 0 |
| | Rats | P1 | 250 | 5 S* | 1:50 S |
| | | P2 | 100 | 2 N* | 1:50 N |
| | | P3 | 100 | 0 | 0 |
| | | P5 | 125 | 1S,1 N | 1:125S,1:125 N |
| | | P6 | 84 | 1 N | 1:84 N |
| | | D1 | 63 | 0 | 0 |
| | | D2 | 63 | 1 S | 1:63 S |
| | | D3 | 28 | 1 S | 1:28 S |
| | | D8 | 21 | 0 | 0 |
| | | D9 | 63 | 0 | 0 |
| | | D10 | 100 | 1 S | 1:100 S |
| Jan/Feb 1977 | Mice** | P1 | 28 | 1 | 1:28 |
| | | P3 | 52 | 10 | 1:5 |
| | | P4 | 63 | 12 | 1:5 |
| | | P5 | 63 | 3 | 1:21 |
| | | P6 | 42 | 0 | 0 |
| | | P7 | 42 | 3 | 1:14 |
| | | D5 | 42 | 1 | 1:42 |
| | | D6 | 63 | 4 | 1:16 |

Table 14.1 cont.

| Date | Species | Trap location | Trap- nights | No.caught | Trap-night average |
|------|---------|---------------|--------------|-----------|---------------------|
| June 1977 | Mice | D3 | 8 | 0 | 0 |
| | | D4 | 32 | 2 | 1:16 |
| | | D5 | 42 | 0 | 0 |
| | | D6 | 84 | 5 | 1:17 |
| | | D7 | 42 | 1 | 1:42 |
| | | D9 | 12 | 0 | 0 |
| | Rats | D4 | 124 | 3 N | 1:41 |
| | | D5 | 112 | 4 N | 1:28 |
| | | D6 | 63 | 0 | 0 |
| | | D7 | 84 | 1 N | 1:84 |
| | | D8 | 56 | 0 | 0 |
| | | D9 | 28 | 0 | 0 |
| | | D11 | 42 | 0 | 0 |
| Nov/Dec 1977 | Mice | P3 | 36 | 4 | 1:9 |
| | | P4 | 36 | 5 | 1:7 |
| | | P5 | 32 | 1 | 1:32 |
| | | P6 | 22 | 1 | 1:22 |
| | | P7 | 22 | 0 | 0 |
| | | D5 | 24 | 1 | 1:24 |
| | | D6 | 24 | 0 | 0 |
| | Rats | P1 | 84 | 1 S | 1:84 |
| | | P2 | 56 | 1 N | 1:56 |
| | | P3 | 84 | 0 | 0 |
| | | P5 | 21 | 1 S | 1:21 |
| | | P6 | 7 | 0 | 0 |
| | | D4 | 56 | 0 | 0 |
| | | D5 | 56 | 0 | 0 |

* S = ship rat

* N = Norway rat

**Mice from this time on were trapped in "Katch-All"traps only

1 stoat, 29 grey ducks and 29 mallard ducks were captured during the four trapping periods. Kidneys were cultured from all specimens. The trap-night averages for different trapping locations revealed that mice were the most common rodents inhabiting the farm buildings and the highest numbers were present in the weaner and grower houses at the piggery. Only two ship rats were trapped in buildings, however shelter belts supported high numbers compared with other rodent species. A focus of Norway rat infestation occurred in the calf battery and adjacent sawdust pit at those times of the year when calves were being grain-fed and eight rats were trapped in this area. No ship rats were trapped in this Norway rat focus. Only three Norway rats were trapped on pasture and in shelter belts, two of which were captured alongside the effluent drain running from the piggery.

Seven possums were trapped in shelter belts during the first trapping period in June and July 1976. Only one possum was trapped during subsequent trapping periods of equivalent trap-nights and it was considered that this species was trapped to extinction. Although at least five rabbits were known to inhabit the dairy farm paddocks alongside the Manawatu River, only two were shot. Thirteen hedgehogs were caught by spotlighting over all areas of the piggery and dairy farm. Ducks were trapped only in the immediate vicinity of the piggery.

Serological evidence of leptospiral infection was found in sera from all species except feral cats, rabbits and the stoat (Tables 14.3 to 14.6). Titres against *ballum* antigen occurred in 3 of 35 mice from which sera was available (9%) and the maximum titre was 1:192. One adult mouse trapped in a shelter belt on the dairy farm had a *pomona* titre of 1:48. No titres were detected against other antigens.

*Ballum* titres were present in three of eight sera from ship rats (38%). One male ship rat trapped at the piggery had a titre of 1:24 to *tarassovi* and a titre of 1:24 to *copenhageni* was recorded in a bacteriologically-positive rat with a *ballum* titre of 1:96. The only titre detected in sera from Norway rats was 1:24 to *ballum* in a rat trapped in the milking shed yards on the dairy farm.

Table 14.2 : Trap-night averages for rodents trapped in farm buildings and on farmland at the Massey Piggery Research Centre and Number 1 Dairy Farm.

| Trapping area | Mice * | | | Ship rats | | | Norway rats | | |
|---|---|---|---|---|---|---|---|---|---|
| | trap-nights | No.caught | T.N.A.** | trap-nights | No.caught | TNA | trap-nights | No.caught | TNA |
| Piggery buildings | 420 | 39 | 9.3 | 421 | 2 | 0.5 | 421 | 2 | 0.5 |
| Dairy farm buildings and yards | 353 | 14 | 4 | 495 | 0 | 0 | 495 | 8 | 1.6 |
| Pasture and shelter belts | 48 | 1 | 2 | 1023 | 9 | 0.9 | 1023 | 3 | 0.3 |

* excludes trapping data for mice in initial survey in June and July, 1976.

** T.N.A. = trap-night average per 100 trap-nights.

Twelve hedgehogs were examined serologically and six had *ballum* titres (50%). Heterologous cross-reactions to other antigens (discussed in Chapter XIII) were present in two sera with *ballum* titres. Hebdomadis serogroup titres were present in three of eight possum sera (35%).

A titre of 1:96 to *pomona* was present in the serum from one of 29 grey ducks (3%). Sera from mallards were negative.

Leptospiral isolates were cultured from house mice, ship rats, hedgehogs and possums (Tables 14.3 to 14.6). All isolates from rodents and hedgehogs were typed as belonging to the Ballum serogroup and one isolate from a mouse was typed at the W.H.O. Reference Laboratory in Atlanta, U.S.A. as being serovar *ballum*. Three isolates obtained from possums were typed as belonging to the Hebdomadis serogroup and haemolysin and absorbed antisera testing of these three isolates (Chapter X) gave results characteristic of serovar *balcanica*. One isolate was typed by cross-agglutination absorption at the W.H.O. Reference Laboratory in Atlanta, U.S.A. as serovar *balcanica*.

The bacteriological prevalence of Ballum serogroup infection in house mice, ship rats and Norway rats, was 16%, 36% and 23% respectively (Table 14.7). The ratios of serological to bacteriological prevalence for house mice and ship rats were 1:2 and 3:4 respectively and these results reinforced the hypotheses put forward in Chapters XII and XIII that these species are maintenance hosts for leptospires of the Ballum serogroup in New Zealand. The ratio of serological to bacteriological prevalence of Ballum serogroup infection in hedgehogs was 6:3 and thus this species is a less efficient maintenance host for these organisms. A similar ratio (2:1) was reported for the hedgehog in Chapter XIII.

No isolations were made from feral cats, rabbits, ducks or the stoat.

*Ballum* infection was present in wildlife throughout the study area (Figure 14.2). Analysis of relative density of mice and ship rats (derived from trap-night averages) and prevalence of infection

Table 14.3 : Serological and cultural results for animals trapped in June and July, 1976.

| Trap Location | Species | Sex | Weight (g) | reciprocal of titre | | | | | | Culture | Serogroup |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *balcanica* | *ballum* | *copenhageni* | *hardjo* | *pomona* | *tarassovi* | | |
| P1 | Possum | F | 2870 | 192 | 0 | 0 | 768 | 0 | 0 | + | Hebdomadis |
| P1 | " | M | 2200 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P1 | " | F | 2950 | 768 | 0 | 0 | 3072 | 0 | 0 | + | " |
| D3 | " | F | 1640 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D3 | " | F | 2250 | 1536 | 0 | 0 | 3072 | 0 | 0 | + | " |
| D10 | " | M | 1050 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D9 | " | F | 1750 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P2 | Norway rat | F | 240 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P2 | " | F | 145 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P6 | " | M | 130 | NT | NT | NT | NT | NT | NT | − | |
| P5 | " | F | 520 | " | " | " | " | " | " | − | |
| P1 | Ship rat | F | 110 | 0 | 0 | 0 | 0 | 0 | 0 | + | Ballum |
| P1 | " | F | 120 | 0 | 96 | 0 | 0 | 0 | 0 | + | " |
| P1 | " | F | 75 | NT | NT | NT | NT | NT | NT | − | |
| P1 | " | M | 175 | 0 | 24 | 0 | 0 | 0 | 0 | + | " |
| P1 | " | F | 165 | 0 | 0 | 0 | 0 | 0 | 24 | − | |
| P5 | " | F | 85 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D2 | " | M | 130 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D3 | " | F | 65 | NT | NT | NT | NT | NT | NT | − | |
| D10 | " | M | 120 | " | " | " | " | " | " | − | |
| P1 | House mouse | M | 22 | " | " | " | " | " | " | + | " |
| P3 | " | F | 13 | " | " | " | " | " | " | − | |
| P3 | " | F | 12 | " | " | " | " | " | " | − | |
| P4 | " | M | 18 | " | " | " | " | " | " | − | |

Table 14.3 cont.

| Trap Location | Species | Sex | Weight(g) | reciprocal of titre | | | | | | Culture | Serogroup |
| | | | | *balcanica* | *ballum* | *copenhageni* | *hardjo* | *pomona* | *tarassovi* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| P1 | Stoat | F | 360 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P1 | Feral cat | F | 2125 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P10 | " | M | 3450 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P1 | Hedgehog | M | 750 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P8 | " | F | 700 | 0 | 48 | 0 | 0 | 24 | 0 | − | |
| A | " | F | 670 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P2 | " | M | 720 | 0 | 24 | 0 | 0 | 0 | 0 | + | Ballum |
| E | " | F | 190 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D10 | " | F | 260 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D11 | " | F | 350 | NT | NT | NT | NT | NT | NT | − | |
| D4 | " | M | 910 | 0 | 0 | 0 | 0 | 0 | 0 | + | Ballum |
| − | " | M | 1050 | 0 | 24 | 0 | 0 | 0 | 0 | + | " |
| − | " | F | 480 | 0 | 384 | 48 | 0 | 96 | 24 | − | |
| D8 | " | F | 510 | 0 | 24 | 0 | 0 | 0 | 0 | − | |

Table 14.4 : Serological and cultural results for house mice trapped in January and February,1977

| Trap Location | Sex | Weight (g) | reciprocal of titre | | | | | | Culture | Serogroup |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *balcanica* | *ballum* | *copenhageni* | *hardjo* | *pomona* | *tarassovi* | | |
| P4 | F | 13 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | F | 20 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | M | 13 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | M | 21 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | F | 20 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | F | 14 | 0 | 0 | 0 | 0 | 0 | 0 | + | Ballum |
| P4 | M | 15 | 0 | 96 | 0 | 0 | 0 | 0 | + | " |
| P3 | M | 15 | NT | NT | NT | NT | NT | NT | − | |
| P3 | M | 14 | " | " | " | " | " | " | − | |
| P3 | M | 12 | " | " | " | " | " | " | − | |
| P3 | F | 12 | " | " | " | " | " | " | − | |
| P3 | M | 12 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P3 | M | 12 | NT | NT | NT | NT | NT | NT | − | |
| P4 | F | 26 | 0 | 0 | 0 | 0 | 0 | 0 | + | " |
| P3 | F | 11 | NT | NT | NT | NT | NT | NT | + | " |
| P3 | F | 19 · | 0 | 0 | 0 | 0 | · 0 | 0 | − | |
| P3 | F | 14 | NT | NT | NT | NT | NT | NT | − | |
| P3 | M | 10 | 0 | 0 | 0 | · 0 | 0 | 0 | − | |
| P7 | M | 16 | 0 | 0 | 0 | 0 | 0 | 0 | + | " |
| P7 | F | 19 | NT | NT | NT | NT | NT | NT | + | " |
| P4 | M | 16 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | F | 14 | 0 | 0 | 0 | 0 | 0 | 0 | − | |

Table 14.4 cont.

| Trap Location | Sex | Weight (g) | balcanica | ballum | copenhageni | hardjo | pomona | tarassovi | Culture | Serogroup |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | reciprocal of titre | | | | | |
| P4 | F | 5 | NT | NT | NT | NT | NT | NT | - | |
| P4 | M | 12 | " | " | " | " | " | " | - | |
| P7 | F | 18 | " | " | " | " | " | " | - | |
| P5 | M | 12 | 0 | 0 | 0 | 0 | 0 | 0 | - | |
| P5 | M | 12 | 0 | 0 | 0 | 0 | 0 | 0 | - | |
| P5 | M | 16 | 0 | 48 | 0 | 0 | 0 | 0 | - | |
| P1 | F | 8 | 0 | 0 | 0 | 0 | 0 | 0 | - | |
| D5 | M | 8 | 0 | 0 | 0 | 0 | 0 | 0 | - | |
| D6 | M | 9 | 0 | 0 | 0 | 0 | 0 | 0 | - | |
| D6 | M | 14 | NT | NT | NT | NT | NT | NT | - | |
| D6 | F | 12 | " | " | " | " | " | " | - | |
| D6 | M | 11 | " | " | " | " | " | " | - | |

Table 14.5 : Serological and cultural results for animals trapped in June, 1977

| Trap Location | Species | Sex | Weight (g) | *balcanica* | *ballum* | *copenhageni* | *hardjo* | *pomona* | *tarassovi* | Culture | Serogroup |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | reciprocal of titre | | | | | |
| D6 | House mouse | M | 17 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D4 | " | F | 14 | 0 | 0 | 0 | 0 | 0 | 0 | + | Ballum |
| D4 | " | M | 17 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D6 | " | M | 10 | NT | NT | NT | NT | NT | NT | − | |
| D6 | " | F | 6 | " | " | " | " | " | " | − | |
| D6 | " | M | 9 | " | " | " | " | " | " | − | |
| D6 | " | M | 8 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D1 | " | M | 20 | 0 | 0 | 0 | 0 | 48 | 0 | − | |
| D4 | Norway rat | F | 425 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D4 | " | M | 120 | NT | NT | NT | NT | NT | NT | − | |
| D4 | " | M | 375 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D7 | " | M | 450 | 0 | 24 | 0 | 0 | 0 | 0 | − | |
| D5 | " | F | 510 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D5 | " | F | 120 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D5 | " | M | 170 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D5 | " | F | 210 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D3 | Hedgehog | M | 980 | 0 | 96 | 24 | 0 | 24 | 0 | − | |
| D10 | " | F | 610 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D10 | Rabbit | F | NT | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D10 | " | M | NT | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| D9 | Possum | M | 2050 | 0 | 0 | 0 | 0 | 0 | 0 | − | |

Table 14.6 : Serological and cultural results for animals trapped in November and December, 1977

| Trap location | Species | Sex | Weight(g) | reciprocal of titre | | | | | | Culture | Serogroup |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *balcanica* | *ballum* | *copenhageni* | *hardjo* | *pomona* | *tarassovi* | | |
| P2 | Norway rat | M | 320 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P1 | Ship rat | F | 130 | 0 | 96 | 24 | 0 | 0 | 0 | + | Ballum |
| P5 | " | F | 65 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P3 | House mouse | F | 17 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P3 | " | M | 18 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P3 | " | F | 13 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P3 | " | M | 13 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | " | M | NT | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | " | M | 14 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | " | F | 16 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P4 | " | F | 8 | NT | NT | NT | NT | NT | NT | − | |
| P4 | " | F | 7 | " | " | " | " | " | " | − | |
| P5 | " | M | 12 | 0 | 0 | 0 | 0 | 0 | 0 | − | |
| P6 | " | F | 17 | 0 | 0 | 0 | 0 | 0 | 0 | + | Ballum |
| P7 | " | F | 13 | 0 | 192 | 0 | 0 | 0 | 0 | − | |

indicated that, in general, populations of higher densities experienced a higher prevalence of infection than populations of lower densities. However, when immature mice and rats were excluded from the analysis, this apparent trend was found to be incorrect and the prevalence of infection was not associated with the relative abundance of rodents in different trapping locations.

The characteristics of Ballum serogroup infection in the free-living species sampled were very similar to those described in earlier chapters for the same species inhabiting different ecosystems. The range of titres in mice was 1:48 to 1:192 with a GMT of 1:114. Ship rats had titres ranging from 1:24 to 1:96 and a GMT of 1:60. Only one low titre was found in sera from Norway rats. The range of *ballum* titres in hedgehogs was 1:24 to 1:384 with a GMT of 1:54. Isolations were often made from seronegative mice and ship rats (83% and 25% respectively). One of three culture-positive hedgehogs (33%) was serologically negative.

There was a marked difference in prevalence of infection between juvenile and adult animals (Table 14.8). Only 1 of 23 house mice was bacteriologically positive (4%) whereas leptospires were recovered from 8 of 34 sexually-mature house mice (24%). No isolations were made from the three juvenile ship rats of four juvenile hedgehogs that were trapped. The bacteriological prevalence in mature animals of these species was 50% and 33% respectively.

## Domestic Animals

Infection with serovar *pomona* was found to be endemic in pigs at the Massey Pig Research Centre, with the main focus of infection being in animals 6 to 12 months old (Ryan, 1978). Some groups of pigs in this age-group had a serological prevalence of *pomona* titres exceeding 80%. Bought-in gilts also had a high prevalence of *pomona* infection.

Titres against *ballum* and *hardjo* antigens were detected in a small number of pig sera with *pomona* titres. These titres never exceeded 1:24 and it was considered that they were cross reactions to *pomona* agglutinins. Two sows had persistent titres of 1:24 and 1:48 to *tarassovi*.

Table 14.7 : Summary data for Ballum serogroup infection in free-living species trapped at Massey Piggery and Massey Number 1 Dairy Farm.

| Species | Sex | | | No. sero. positive | | | Sero.prevalence* (%) | No. bact.positive | | | Bact.prevalence (%) | Sero:bact.* prevalence. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | F | Total | M | F | Total | | M | F | Total | | |
| House mouse | 33 | 25 | 58 | 2 | 1 | 3 | 9 | 3 | 6 | 9 | 16 | 1:2 |
| Norway rat | 6 | 7 | 13 | 1 | 0 | 1 | 10 | 0 | 0 | 0 | 0 | – |
| Ship rat | 3 | 8 | 11 | 1 | 2 | 3 | 38 | 1 | 3 | 4 | 36 | 3:4 |
| Hedgehog | 6 | 7 | 13 | 3 | 3 | 6 | 50 | 3 | 0 | 3 | 23 | 6:3 |
| Possum | 6 | 2 | 8 | 0 | 3 | 3 | 38 | 0 | 3 | 3 | 38 | 1:1 |
| Feral cat | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – |
| Stoat | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – |
| Rabbit | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – |
| Grey duck | 8 | 7 | 29** | 1 | 0 | 1 | 3 | 0 | 0 | 0 | 0 | – |
| Mallard duck | 10 | 9 | 29*** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | – |

* calculated for those animals from which sera examined.

** 14 juveniles not sexed.

*** 10 juveniles not sexed.

Table 14.8 : Prevalence of Ballum serogroup infection in juvenile
and mature house mice, ship rats and hedgehogs from
the Massey Pig Research Centre and No. 1 Dairy Farm.

| Species | Prevalence of Ballum serogroup infection (%) | | |
|---------|----------|--------|--------------|
|         | Juvenile | Mature | Whole sample |
| House mouse | 4 | 24 | 16 |
| Ship rat | 0 | 50 | 36 |
| Hedgehog | 0 | 33 | 23 |

Serological results from the No. 1 dairy herd revealed that Hebdomadis serogroup infection was endemic in cattle on the dairy farm (Hellstrom, 1978). *Hardjo* titres were present in 97% of adult cattle. Hiefers were infected from the age of eight months, and *hardjo* was isolated from urine from several of these animals.

*Pomona* titres were found in 31% of cattle sera at the beginning of the study and this had dropped to 18% by the end. These *pomona* titres were low and all occurred in animals more than five years old. Titres of 1:24 to *ballum* were found in seven cows. Titres of the same level to both *tarassovi* and *copenhageni* were found in three cows and one cow respectively. These low titres were recorded only in sera with titres to *hardjo* or *pomona* of ≥ 1:384 and were never recorded in more than one serum from serial samples from any one cow. It was therefore concluded that they were cross-reactions.

### Environmental samples

Leptospires of the Pomona serogroup were recovered from a sample of effluent from the drain running through the centre of the piggery and also from surface water in the gilt paddock (Location A). Hellstrom (1978) recovered an isolate of the same serogroup from a pond that lay in a depression on the dairy farm below the sow and gilt grazing paddocks (location F).

### DISCUSSION

Most of the common small mammalian species of wildlife present on New Zealand farmland were found to inhabit the Massey Pig Research Centre and the No. 1 Dairy Farm. The distribution of each free-living species in different biotopes in the study area was characteristic of the preferred-biotope distribution described in earlier chapters for different ecosystems. Rodents were the most common animals encountered, although their relative abundance fluctuated during the period of the investigation. Factors affecting relative abundance were sporadic poisoning operations by farm staff and seasonal activities such as grain-feeding of calves. Trap-night averages for rodents indicated that population densities were

comparable or higher to those on dairy farms investigated by Brockie (1977). Thus the presence of relatively high numbers of several species of wildlife in an area of intensive pig and dairy farming provided a very suitable ecosystem in which to study the nidality of leptospirosis.

Leptospirosis was endemic in cattle, pigs and wildlife in the study area. Four different serovars were isolated and each serovar formed a reservoir of infection in a particular host species. These maintenance host-parasite relationships were very well defined.

Serogroup Ballum infection was endemic in house mice, ship rats and hedgehogs and prevalences of infection were similar to those found in other environments in New Zealand. These species were undoubtedly the maintenance hosts for these organisms in the ecosystem under investigation. Except for one house mouse, infection was restricted to sexually-mature animals of all species. An age differentiation in prevalence of infection has been noted by workers investigating the same species in other countries (Clark, 1961; Wolff and Bohlander, 1965) and is also evident in the possum in New Zealand. This phenomenon is further discussed in other chapters. It was also found that a high prevalence of Ballum serogroup infection occurred in three free-living species inhabiting the same ecosystem. This situation has not been reported in other countries. A similar situation was described and discussed for a different ecosystem in Chapter XIII.

Trapping data revealed that the relative abundance of rodents in this syanthropic focus was higher than that in natural foci investigated in earlier chapters, and the high prevalence of Ballum infection was maintained despite control measures and subsequent fluctuations in the population density. The lack of a dependance on a high population density to maintain Ballum serogroup infection in free-living populations (other than the Norway rat) and the rapid repopulation of a specific biotope following control operations indicates that a nidus of infection would always be present in the environment being investigated.

Serovar *pomona* was endemic in the pig population. The high prevalence of infection in young pigs and bought-in gilts ensured continued transmission of the disease and *pomona* infection in the pigs was characteristic of leptospiral infection in a maintenance host. No clinical symptoms attributable to *pomona* infection occurred in pigs during the study.

A very high prevalence of *hardjo* infection was found in cows on the dairy farm. Cattle are recognised in New Zealand and other countries as the maintenance hose for *hardjo*. Hiefers with asymptomatic infection were shown to excrete *hardjo* for periods of up to a year (Hellstrom, 1978).

Possums in shelter belts were infected with *balcanica* and this species has been described previously as a maintenance host for this serovar. Thus four, well-defined maintenance host-parasite relationships were found to be present in the intensive farming environment under study.

There was virtually no evidence of cross-species transmission of leptospires throughout the two year duration of the investigation. Clinical records of dairy farm cattle revealed that an outbreak of *pomona* had occurred in the herd five years previously and the *pomona* titres in cattle were all attributable to this outbreak. *Pomona* outbreaks are well-recognised in cattle in New Zealand although there is no evidence that they maintain this serovar (Hellstrom, 1978). Titres to antigens other than *pomona* in pigs and cattle, and *hardjo* in cattle, were low and infrequent and were considered to be cross-reactions (Ryan, 1978; Hellstrom, 1978). It was possible that persistant but low *tarassovi* titres in two sows indicated past infection with this serovar, however no isolates were made.

The only evidence of infection in wildlife with leptospires other than *balcanica* and those of the Ballum serogroup was a titre of 1:48 to *pomona* in a mouse, a titre of 1:24 to *tarassovi* in a ship rat and a titre of 1:96 to *pomona* in a grey duck. Parnas *et al* (1961), summarising leptospirosis in house mice in Europe, reported that *pomona* has been recovered from this species, although it was a very rare occurrence. It is therefore possible that the *pomona* titre in

a mouse on the dairy farm may have represented a past infection with this serovar. Emanuel (1959) experimentally produced leptospiruria in laboratory mice with *pomona*, however Chernukcha *et al* (1974) were unable to repeat these results. The titre to *tarassovi* in a ship rat from the piggery is of unknown significance. Similar titres were detected in sera from two Norway rats shot on the Palmerston North rubbish tip and this serovar has been sporadically isolated from Norway rats in other countries (Anon, 1966). It is therefore possible that rats may be susceptible to infection with *tarassovi* in New Zealand.

The titre of 1:96 to *pomona* in 1 of 58 ducks (1.7%) indicates that ducks may be susceptible to challenge by this organism, however, no isolations were made from liver or kidneys. *Pomona* was isolated from piggery effluent and the feeding behaviour of the ducks ensured maximal challenge under natural conditions. The very low serological prevalence and lack of isolations from ducks inhabiting the piggery demonstrates that they are of no epidemiological significance in the transmission of *pomona*. This finding is in common with reports from workers investigating birds inhabiting foci of endemic leptospirosis in other countries (Torten *et al*,1965). Kenzy and Gillespie (1957) found *pomona* titres in 12 of 20 chickens (60%) on a farm where an epidemic of *pomona* occurred in cattle, however no isolations were made from these or experimentally challenged chickens.

The lack of interspecies transmission of leptospirosis in the intensive farming environment that was studied provides some important information on the nidality of this disease under New Zealand conditions. It was considered that environmental contamination with leptospires and the potential for interspecies transmission were very high because of the following factors :

1.  Endemic infection in the respective maintenance hosts for each serovar was of high prevalence and all species have been shown to shed these leptospires for long periods.

2.  As a consequence of the intensive nature of the farming systems practised in the study area, excretion of leptospires

by domestic animals was concentrated over a small geographical area.

3.   Environmental conditions, and in particular the amount of surface water, were favourable for the survival of lepto-spires.   Water has been widely reported as a vehicle of interspecies transmission of leptospirosis (Babudieri, 1958; Doherty, 1967; Baker and Baker, 1970, Buddle  and Hodges 1977) and *pomona* was recovered from piggery effluent and surface water in paddocks.   No other serovars were recovered by environmental sampling. *Pomona* has often been recovered from water in the U.S.A. (Gillespie, 1957; Gillespie and Ryno,1963) and *ballum* has also been recovered from surface water in an environment where endemic *ballum* infection occurred in house mice (Clark, 1961).

4.   The geographical distribution of species infected with different serovars involved considerable overlap (Figure 14.2). Domestic animals grazed on adjoining paddocks, some of which had common drainage, and wildlife were present on pasture and in farm buildings and grain stores.   Rodents were even seen in pens with calves feeding out of calf meal buckets.

There are three important epidemiological factors which must be evaluated when considering the lack of interspecies transmission in the face of this widespread environmental contamination and an apparent high potential for transmission.   These are the resistance of different species to challenge with a particular serovar, species behaviour and ability of different serovars to survive in the environment.

1.*The resistance of different species to challenge with a particular serovar.*

It has been well documented by various workers that different animal species have widely different susceptibilities to experimental challenge with different serovars (Emanuel,1959; McGowan and Karstad,1965; Stalheim,1966a;Reilly,1970) and some species are resistant to challenge with certain serovars

Figure 14.2. : Geographical distribution of leptospirosis in domestic animals at the Massey Pig Research Centre and No.1 Dairy Farm.

hardjo
pomona
ballum
balcanica
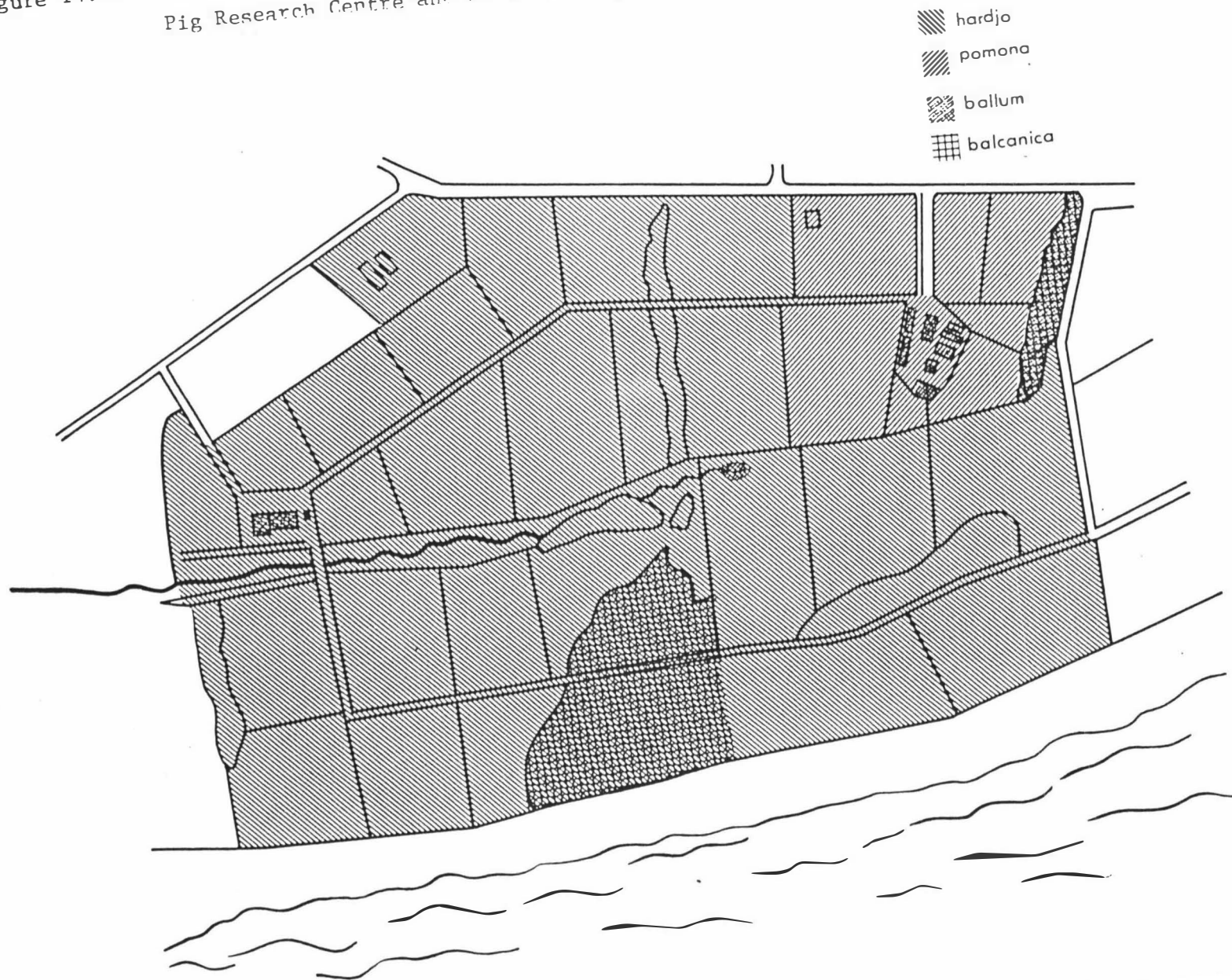
(Chernukcha *et al*,1974). Woods *et al* (1962) failed to isolate *ballum* from 18 experimental-challenged pigs and the titres that were detected in seven pigs were low and transient. In contrast, calves have been successfully infected by experimental challenge with *ballum* (Anon,1973).

*2.Species behaviour.*

Host behaviour may limit or enhance the possibility of a particular species contacting the infectious agent. For example, house mice prefer a dry environment and therefore, although they are common in piggery buildings, they would not come into contact with effluent which is considered to be an important vehicle for the transmission of *pomona*. Norway rats on the other hand frequently inhabit wet biotopes and therefore would probably have considerable contact with effluent at the piggery (several were trapped within the immediate area of the effluent drains).

*3.Ability of different serovars to survive in the environment.*

Direct contact is probably the most important factor in the intraspecies transmission of leptospirosis. However different species are unlikely to have the contact necessary for direct transmission. Therefore, indirect transmission via the environment is of more importance. *Pomona* is known to survive for at least 6 weeks outside of an animal host (Hellstrom, 1978), however little is known about the survival of *ballum, balcanica* or *hardjo* in the environment. It is possible that at least for *hardjo*, environment survival is short as this serovar is difficult to grow in artificial media compared with other serovars. It is also very difficult to recover from material containing other micro-organisms. Therefore, although environmental contamination by leptospiruric cattle may be high, a short survival time of shed organisms would mean that the potential for interspecies transmission of *hardjo* was low. It is of interest in the present study that the only evidence of interspecies transmission of leptospirosis was with *pomona*, an organism known to survive for long periods in the environment.

Although there was no bacteriological and very little
serological evidence of interspecies transmission in the present
investigation the results must be interpreted with some degree
of caution. Leptospiral infection in an accidental host usually
results in only low and transient titres (Chernukcha *et al*,1974)
and a short renal phase of infection. Cross-sectional sampling
of wildlife may fail to detect a low prevalence of accidental
infections, as may serological sampling of domestic animals at
four month (or longer) intervals. The low antigenicity of *ballum*,
that has been discussed in earlier chapters, may be especially
important in this respect. The very similar serological cross-
reactivity of *hardjo* and *balcanica* agglutinins also presents
problems when attempting to determine the occurrence of cross-species
transmission of these serovars by serological techniques. However,
if sporadic and undetected infections do occur outside the described
maintenance host-parasite relationships, they are unlikely to be of
any epidemiological significance in the maintenance of a particular
nidus.

The results of the present investigation for evidence of inter-
species transmission of leptospirosis support the general survey data
available for the same species in different farming areas of New
Zealand. The distribution of serovars in wildlife has been discussed
in earlier chapters. Ballum serogroup infection appears to be rare
in cattle with very low serological and bacteriological prevalences
having been reported (see Chapter I) and *ballum* has not been isolated
from pigs. The sporadic Ballum serogroup infections in cattle are
most likely due to transmission from wildlife. No such transmission
was demonstrated in the present study, however several workers in
other countries have described concurrent *ballum* infection in cattle
and house mice sharing a specific ecosystem (Clark, 1961; Martin
*et al*, 1967; Schnurrenberger *et al*, 1970). *Ballum* infection is very
rare in pigs in other countries. Kmety *et al* (1956) isolated *ballum* from
1 of 460 pigs in Slovakia (0.2%) and Chung (1968) reported only 1 of
281 sera from pigs in Australia to be sero-positive to *ballum* at a
minimum dilution of 1:100.

The nidality of other serovars investigated in the study area
was different to that reported in other countries. *Pomona* is often

present in both wildlife and domestic animals sharing a specific ecosystem and interspecies transmission of this serovar in both directions has been suggested by several workers, (Blood *et al*, 1963; Roth *et al*, 1964; Mitchell *et al*, 1966; Fennestad and Borg-Petersen, 1972). MacGowan and Karstad (1965) successfully transmitted *pomona* from ground hogs (*Marmota monax*) to cattle and pigs via infected drinking water. *Balcanica* has been isolated from pigs, cattle and humans in East Europe but no wildlife reservoirs have been identified (Matveeva *et al*, 1977). This is in contrast to the situation that is recognised in New Zealand.

*Copenhageni* was not present in the ecosystem under study. It is likely that if this serovar had been endemic in Norway rats inhabiting the piggery and dairy farm, inter-species transmission may have been demonstrated. Mice can act as accidental hosts for *copenhageni* (Alexander *et al*, 1963; Roth, 1970; Urquhart, 1973) and this serovar is often transmitted from Norway rats to domestic stock and man. It is interesting to note that Norway rats from the piggery were all bacteriologically negative, despite an environment heavily contaminated with *pomona*. This species appears to be resistant to *pomona* infection, although it is an excellent maintenance host for *copenhageni*.

The finding of major importance in the present investigation was the very well defined maintenance host-parasite relationships that were maintained in the face of a widespread potential for interspecies transfer. This illustrated the natural nidality of leptospirosis in an intensive farming environment. It was well demonstrated that the serovars of major public health significance in New Zealand, *hardjo* and *pomona*, were independently maintained in domestic animals, and *ballum* and *balcanica* infection was restricted to wildlife. *Ballum* infection in humans in New Zealand is very rare and the occurrence of *balcanica* infection in humans has yet to be determined. Brockie (1977) suggested that humans were most probably infected with *ballum* via the agency of cattle. In light of the results of the present work, it is more likely that humans are infected via direct or indirect contact with wildlife. The general situation in New Zealand is considerably different from that in other countries where serovars of public health importance are often maintained by free-living species.

## SUMMARY AND CONCLUSIONS

1.   A variety of potential free-living carriers of leptospirosis were found to be present in an intensive pig and dairy farming ecosystem.

2.   Four of the six serovars that have been isolated in New Zealand were found to be endemic in different host species : *ballum* in house mice, shiprats and hedgehogs; *balcanica* in possums, *hardjo* in cattle and *pomona* in pigs.

3.   Very well defined maintenance host-parasite relationships were demonstrated in each animal species.

4.   Norway rats, ducks, rabbits and feral cats were bacteriologically negative.

5.   There was no bacteriological and very little serological evidence of interspecies transfer of leptospires during the two year study period.   Retrospective evidence of a *pomona* outbreak that occurred five years previously was detected in cattle sera.

6.   *Pomona* was recovered on three occasions from environmental samples.

7.   The nidality of leptospirosis in the ecosystem under study is discussed in terms of the epidemiological factors influencing inter-species transfer.

8.   The study established that the two most important serovars in New Zealand from a public health point of view, *hardjo* and *pomona*, were maintained independently in domestic animals.

CHAPTER XV

THE MAINTENANCE HOST

INTRODUCTION

The original concept of a maintenance host for an infectious
agent was put forward by Audy (1958). He described a maintenance
host as an animal which carried a particular agent for long enough
to allow transmission of infection from generation to generation.
Babudieri (1958) noted that in leptospiral infection of rodents,
some serovars exerted only a minimal pathological effect on the
tubular epithelium during the renal carrier phase and he considered
such serovars to be in a state of "biological equilibrium" with
their hosts. These rodents were categorised as reservoir hosts for
particular serovars.

Roth *et al* (1963) extended the concept of maintenance hosts
for particular serovars to explain particular host-parasite
relationships which occurred in skunks infected with a variety of
serovars in the U.S.A. These workers established serological and
bacteriological criteria which served to define leptospiral infection
in a maintenance host. Several subsequent workers have applied these
criteria to field data to identify maintenance hosts for particular
serovars in specific ecosystems (Torten *et al*, 1970; Fennestad and
Borg-Petersen, 1972; Chernukcha *et al*, 1974).

It has been established that the pathogenicity of a particular
serovar varies for different animal species (McGowan and Karstad, 1965;
Stalheim, 1966a; Reilly, 1970) and that different serovars vary in
pathogenicity for a particular animal species (Emanuel, 1959; Chernukcha
*et al*, 1974). Such experimental investigation is necessary to
confirm whether a particular free-living species is a maintenance
or accidental host for a specific serovar. By determining the
characteristics of infection in an animal species, the potential
role of that species in the nidality of leptospirosis in a specific
ecosystem can be better established.

Previous chapters have described endemic *balcanica* infection

in possums and endemic Ballum serogroup infection in house mice, ship
rats and hedgehogs. Experimental infections in possums confirmed
that this species was a maintenance host for *balcanica* and serological
and bacteriological evidence of Ballum serogroup infection in other
species, according to the criteria of Roth *et al* (1963), was
characteristic of leptospirosis in a maintenance host. The house
mouse has also been shown to be a maintenance host for *ballum* in
other countries (van der Hoeden, 1958; Clark, 1961).

This chapter describes the use of laboratory mice as an
experimental model to determine the relative pathogenicity of
*ballum, balcanica, pomona* and *hardjo* in this species and as a
consequence define the characteristics of leptospiral infection in
a maintenance host. The criteria used by previous workers to
define leptospiral infection in a maintenance host compared with
an accidental host have been relative pathogenicity in clinical
terms (Emanuel, 1959), characteristics of leptospiruria and
ratio of serological to bacteriological prevalence in a population
(Roth *et al*, 1963) and age susceptibility and immunological response
to infection (Chernukcha *et al*, 1974; 1975). In this study, these
criteria, as well as the infective dose of a particular serovar and
the demonstration of intraspecies transmission, are investigated
in a series of experiments in a laboratory mouse model. The model
is also used to investigate age differences in the prevalence of
infection in a population. By combining the experimental results
with field data from maintenance host populations, the concept of
a maintenance host is redefined in terms of individual animals and
maintenance populations.

## MATERIALS AND METHODS

Endemic leptospiral infection has been reported in laboratory
mouse colonies in the U.S.A. and Europe (Stoenner *et al*, 1958;
Kemenes and Szeky, 1966). A random sample of 30 mice from the
Massey University Laboratory Animal Unit were screened serologically
and culturally for evidence of leptospirosis. Serogroup Ballum
infection was found to be endemic in the colony, with isolates being
obtained from four of ten adult mice (40%). Three adults (30%) and
one weanling mouse (5%) had *ballum* titres ranging from 1:24 to 1:192.

(No titres were detected against *copenhageni, hardjo, pomona* and *tarassovi* antigens). As a consequence, stock mice for experimental investigations were obtained from a specific pathogen free (SPF) colony at the Auckland Medical School. No evidence of leptospiral infection was found in these animals.

The *ballum, balcanica, hardjo* and *pomona* isolates used to inoculate experimental mice had been isolated respectively from a house mouse, possum, cow and pig from the Massey Pig Research Centre and Massey Number 1 Dairy Farm. These isolates were identified by cross-agglutination absorption at the WHO Reference Laboratory, Atlanta, U.S.A.

Several workers have reported a loss in virulence of leptospiral cultures serially passaged in liquid media (van Thiel, 1948; Stalheim, 1966b; Tripathy *et al*, 1974). Other workers have found little loss in virulence of isolates passaged for periods of up to 12 months (Ellinghausen and Painter, 1976). The isolates used in the present study had been serially passaged for more than a year in liquid media. To ensure that the isolates had a similar potential to infect mice, they were first serially passaged in weanling hamsters. Each serovar was inoculated initially into three hamsters and these were observed daily for clinical symptoms. Kidneys from dead or comatose hamsters were homogenised using the syringe technique described in Chapter V and one ml of homogenate was inoculated IP into one of another group of three hamsters. At the termination of the hamster passages, kidney homogenates were cultured in semisolid EMJH and primary isolates were subcultured into liquid EMJH. The density of liquid cultures used for inoculation of experimental mice was established by the mean of three counts in a Petroff-Hauser counting chamber.

Experiment 1: Infective dose, clinical response and serological and bacteriological parameters of *ballum, balcanica, hardjo* and *pomona* infection in mice.

Liquid cultures of the above serovars were counted in a Petroff-Hauser chamber and serial ten-fold dilutions from $10^8$ to $10^1$ organisms/ml were made in liquid medium. One ml of each culture dilution was inoculated IP into each of a group of six laboratory

mice (mean weight 23.9 ± 2.1 g). Four groups of six mice were inoculated with media and maintained as controls. The virulence of each serovar was titrated by IP inoculation of $10^8$ organisms into groups of four hamsters. Dilutions of each serovar used for mouse inoculations were cultured back in liquid media.

Inoculated mice were observed daily for clinical symptoms. The experiment was terminated at 17 days p.i. so as to minimise the chance of secondary spread of infection within treatment groups. All mice were anaesthetised with ether and bled by cardiac puncture. Sera were tested against *ballum*, *balcanica*, *hardjo* and *pomona* antigens at a minimum serum dilution of 1:12. Kidneys were cultured as previously described.

Experiment 2 : Characteristics of leptospiruria and long-term kidney infection in mice inoculated with *ballum*, *balcanica*, *hardjo* and *pomona*.

The 48 mice that were inoculated with different numbers of each serovar in Experiment 1 were monitored by dark-field microscopy at days 11, 14 and 17 p.i. Urine was collected on microscope slides and one bacteriological loop of urine was diluted with one loop of physiological saline before examination. The intensity of shedding was scored as shown in Table 15.1. The motility of the leptospires was also noted.

Table 15.1 : Scoring system for intensity of leptospiruria in experimentally-infected mice.

| No. leptospires per dark field | Score |
|---|---|
| 0 | 0 |
| <1 | 1 |
| ≥1  <10 | 2 |
| ≥10  <30 | 3 |
| >30 | 4 |

A second part of this experiment involved the monitoring of urine from chronically infected mice for a period of six months p.i. Groups of six mice were inoculated with $10^8$ leptospires of each serovar as in Experiment 1. Leptospiruria was monitored by dark-field microscopy at weekly intervals for the first four weeks p.i. and then at monthly intervals. At the termination of this experiment, kidneys were cultured from all mice and sera were tested against the infecting serovar at a minimum serum dilution of 1:12. The sensitivity of dark-field microscopy relative to kidney culture for the detection of leptospiral infection with the four serovars used in this experiment was also determined.

### Experiment 3 : Infectivity of leptospires shed in mouse urine and natural transmission of *ballum*, *balcanica*, *hardjo* and *pomona*.

This experiment was carried out to determine if leptospiral infection in mice with the above-mentioned serovars was artificially or naturally transmissible to the same species. Urine samples were collected from each of groups of two mice that had been inoculated with the respective serovars 21 days previously. These were examined by dark-field microscopy and also cultured according to standard techniques. Each of three mice was then inoculated with 0.2 ml of each urine sample. Kidneys from these mice were cultured at 21 days p.i.

Natural transmission was investigated by the introduction of three males into cages containing three females that had been inoculated eight weeks previously with the above-mentioned serovars. The urines from the female mice were examined by dark-field microscopy at the time of introduction of the males. Urine from male mice was examined at four weeks and six weeks after introduction to the female mice and kidneys from all mice were cultured at eight weeks after introduction.

### Experiment 4 : Age susceptibility of mice to infection with *ballum*, *balcanica*, *hardjo* and *pomona*.

Six groups of three adult mice (mean weight 33.4 $\pm$ 4.5 grams) were inoculated with $10^1$, $10^2$, $10^3$, $10^4$, $10^6$ and $10^8$ *ballum* leptospires by the IP route and this was repeated using *pomona*. The

same procedure was applied to weanling mice (mean weight 7.7 $\pm$ 1.2
grams). The age susceptibility to *balcanica* and *hardjo* was invest-
igated using four groups of three adult mice inoculated with $10^3$,
$10^4$, $10^6$ and $10^8$ leptospires and this procedure was repeated using
weanling mice. Two groups of three adult mice and two groups of
three weanling mice were maintained as controls. All dilutions
of inoculating serovars were cultured back in liquid media and
the virulence of each serovar was titrated in groups of three
weanling hamsters inoculated with $10^8$ organisms. Kidneys from all
mice were cultured according to standard techniques at 17 days p.i.

Experiment 5 : An investigation of differences in age prevalence
of infection using a laboratory mouse model.

Field studies demonstrated a marked difference in prevalence of
Ballum serogroup infection between sexually-immature and mature
house mice. Similar findings have been reported in endemic
leptospiral infection in a variety of free-living species serving
as maintenance hosts for a particular serovar and it has been
suggested that this difference in age prevalence of infection is
due to a venereal route of transmission (see Chapters VI and
VIII). Such a route of transmission would restrict infection to
sexually-mature animals only.

The first part of this experiment was designed to investigate
the role of maternal antibody and the role of a venereal route of
transmission as determinants of an age difference in prevalence
of infection. The role of maternal antibody was investigated by
inoculation of three groups of five mice with 0.5 ml of a $10^8$
*ballum* culture by the IP route. Each of the three groups of mice
were from litters from dams that were shedding *ballum* in their urine
at the time of parturition and subsequent to this. Group 1 mice were
two weeks of age, group 2 mice were four weeks of age and group 3
mice were eight weeks of age. Groups 2 and 3 had been removed from
their dams at four weeks of age. Littermates that were not
inoculated with *ballum* served as controls. All mice were cultured
at 17 days p.i.

For an investigation of venereal transmission, ten male and ten female adult mice were infected with *ballum* and all were determined to be leptospiruric by dark-field microscopy at day 16 p.i. Each mouse was then individually caged and ear-clipped and one non-infected mouse was introduced to each cage according to the protocol detailed in Table 15.4. This involved infected males with non-infected females and vice-versa and also involved same-sex pairs of infected and non-infected mice. Four pairs of controls were maintained. After a period of four weeks, urine from all mice was examined by dark-field microscopy and kidneys were cultured according to standard techniques.

A second part of the experiment investigating venereal transmission involved the infection of newborn mice with *ballum* and the surveillance of litter-mates and dams to determine the transmission pattern of leptospirosis. Three, two week old males in each of two litters ( 9 and 10 mice respectively) were inoculated with 0.5 ml of a $10^8$ organisms/ml culture of *ballum*. Urine from infected males, litter-mates and dams were monitored according to the schedule shown in Figures 15.5 and 15.6. The experiment was terminated when the young mice were 60 days of age; kidneys were cultured according to standard techniques. This experiment was duplicated using two litters of mice (8 and 10 respectively) and *pomona*.

## Experiment 6 : Ratios of serological and bacteriological prevalence for the respective serovars.

Data to determine these ratios was taken from Experiment 1 and Experiment 2. The duration of Experiment 1 was 17 days and therefore this experiment provided both serological and bacteriological prevalences for short-term infection. Chronically-infected mice in Experiment 2 were maintained for six months and therefore the ratio of serological and bacteriological prevalence for each serovar in these mice was based on long-term infection.

## RESULTS

Death times of hamsters serially infected with different serovars

showed a small degree of fluctuation (Figure 15.1). The range of
death times was 5 to 7 days for *ballum*, 4 to 8 days for *pomona* and
11 to 15 days for *balcanica*. Mean death times and standard deviations
for the respective serovars were 5.55 $\pm$ 0.60 days, 5.53 $\pm$ 0.92 days
and 13.1 $\pm$ 1.29 days. Infection was not transmitted after the
second passage with *pomona* in one hamster and the first passage
with *balcanica* in one hamster. Hamsters inoculated with *hardjo*
showed no clinical symptoms and therefore kidney homogenates were
passaged into another group of hamsters after three weeks. Kidney
homogenates from this group were cultured after three weeks and
*hardjo* isolates were recovered from two of the three hamsters.
These isolates required two passages in liquid media to reach a
density of $10^8$ organisms/ml.

Experiment 1 : Infective dose, clinical response and serological
and bacteriological parameters of infection with *ballum, balcanica,
hardjo* and *pomona*.

Clinical symptoms were not observed in any mice inoculated with
the four serovars. This was in marked contrast to the situation
in hamsters inoculated with the same cultures. Hamsters infected
with *ballum* and *pomona* had mean death times of 5.25 and 4.75 days
respectively. Hamsters infected with *balcanica* showed characteristic
central nervous system symptoms (see Chapter V) and had a mean death
time of 13.25 days. No clinical symptoms were observed in hamsters
inoculated with *hardjo*.

The serological and cultural results of mice inoculated with
different numbers of leptospires of each serovar are given in
Table 15.2. The minimum infective dose (MID) for *ballum* and *pomona*
was ten organisms. Infection with *ballum* within groups of mice
was very consistent, with all mice in all treatment groups being
culture-positive at day 17 p.i. This also demonstrated the efficiency
of the cultural technique for recovering *ballum* from infected mice.
Three of six mice receiving $10^1$ *pomona* organisms (50%) and one of
six mice receiving $10^2$ *pomona* organisms (17%) were bacteriologically
negative.
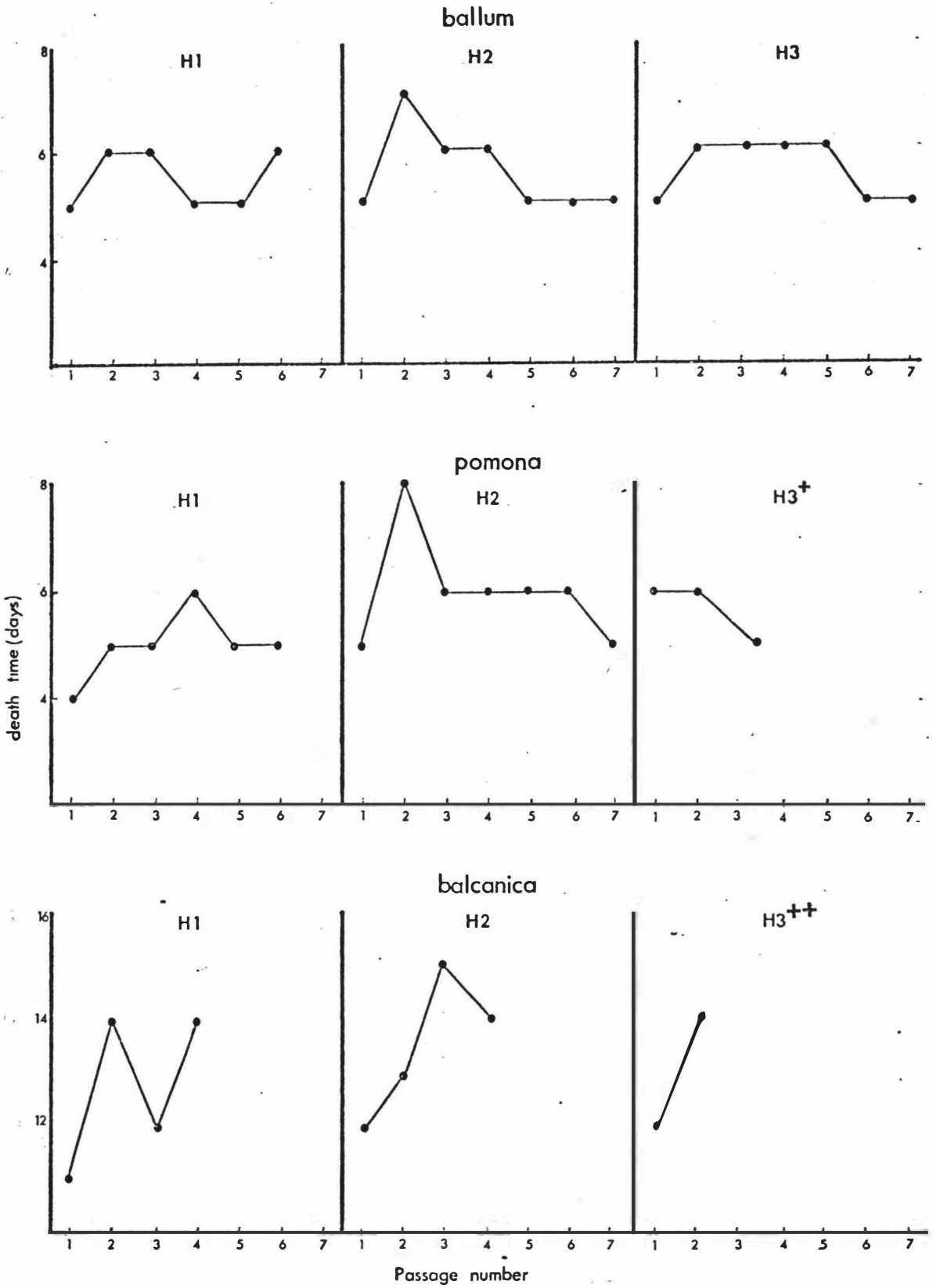
Figure 15.1. : Serial passage of *ballum, pomona* and *balcanica* in
weanling hamsters.

+ infection not transferred after second passage
++ infection not transferred after first passage

H = Hamster

The MID for *balcanica* and *hardjo* was $10^7$ organisms. Infection with these serovars within groups was inconsistent (Table 15.2), with $\leqslant 50\%$ of mice within groups receiving $10^7$ or $10^8$ organisms becoming infected.

Serological results (Table 15.2) revealed that *ballum* titres in infected mice were very low and the GMT of positive sera was 1:29. Isolations were made from 27 mice that had no titres (56%). There was no correlation between level of titre in infected mice and number of organisms in the inoculum.

*Pomona* titres were considerably higher than *ballum* titres. Mice infected with *pomona* had a GMT of 1:285 for positive sera. No isolations were made from seronegative animals. As with mice infected with *ballum* there was no correlation between level of titre and number of infecting organisms.

Serological evidence of *balcanica* infection was found in mice receiving $10^3$ to $10^8$ leptospires, although isolations were limited to mice receiving $10^7$ and $10^8$ leptospires. *Hardjo* titres were present only in mice inoculated with $10^7$ or $10^8$ organisms. Titres to both these Hebdomadis serogroup leptospires were low (GMT's for positive sera of 1:54 and 1:81 respectively) and 11 of 17 mice with *balcanica* titres (65%) and 3 of 4 mice with *hardjo* titres (75%) were bacteriologically negative. No isolations were obtained from seronegative mice.

No cross-reactions were observed in sera from mice infected with *ballum* or *pomona*. Cross-reactivity occurred in sera of mice infected with *balcanica* and *hardjo,* however, titres to the homologous antigen were higher or equal to those against the heterologous antigen.

There was no serological or bacteriological evidence of leptospiral infection in control groups of mice. Leptospires were cultured back from all dilutions of the respective serovars used as inocula except the $10^1$ dilution of *hardjo*.

Experiment 2 : Characteristics of leptospiruria and long-term kidney infection.

Table 15.2 : Serological and cultural results of mice inoculated with different doses of *ballum*,
*balcanica, hardjo* and *pomona*.

| Inoculum | Mouse number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | |
| | titre | cult. | titre | cult. | titre | cult. | titre | cult. | titre | cult. | titre | cult. |
| $10^1$ *ballum* | 0 | + | 0 | + | 0 | + | 0 | + | 0 | + | 0 | + |
| $10^2$ | 0 | + | 0 | + | 0 | + | 0 | + | 0 | + | 0 | + |
| $10^3$ | 24* | + | 0 | + | 0 | + | 12 | + | 0 | + | 0 | + |
| $10^4$ | 12 | + | 24 | + | 24 | + | 12 | + | 0 | + | 12 | + |
| $10^5$ | 12 | + | 12 | + | 12 | + | 0 | + | 0 | + | 12 | + |
| $10^6$ | 12 | + | 0 | + | 12 | + | 12 | + | 0 | + | 0 | + |
| $10^7$ | 0 | + | 0 | + | 12 | + | 0 | + | 24 | + | 12 | + |
| $10^8$ | 24 | + | 0 | + | 0 | + | 12 | + | 24 | + | 12 | + |
| $10^1$ *pomona* | 0 | − | 96 | + | 0 | − | 0 | − | 96 | + | 96 | + |
| $10^2$ | 192 | + | 384 | + | 192 | + | 192 | + | 0 | − | 192 | + |
| $10^3$ | 192 | + | 192 | + | 96 | + | 96 | + | 192 | + | 96 | + |
| $10^4$ | 192 | + | 48 | + | 384 | + | 48 | + | 96 | + | 96 | + |
| $10^5$ | 96 | + | 384 | + | 96 | + | 192 | + | 384 | + | 48 | + |
| $10^6$ | 96 | + | 96 | + | 192 | +· | 192 | + | 96 | + | 384 | + |
| $10^7$ | 768 | + | 96 | + | 96 | + | 96 | + | 48 | + | 96 | + |
| $10^8$ | 192 | + | 384 | + | 96 | + | 48 | + | 384 | + | 192 | + |

Table 15.2 cont.

| Inoculum | Mouse number | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | |
| | titre | cult. | titre | cult. | titre | cult. | titre | cult. | titre | cult. | titre | cult. |
| $10^1$ *balcanica* | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − |
| $10^2$ | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − |
| $10^3$ | 0 | − | 24 | − | 0 | − | 0 | − | 0 | − | 12 | − |
| $10^4$ | 0 | − | 0 | − | 48 | − | 0 | − | 48 | − | 12 | − |
| $10^5$ | 0 | − | 0 | − | 0 | − | 12 | − | 0 | − | 0 | − |
| $10^6$ | 24 | − | 12 | − | 0 | − | 12 | − | 0 | − | 0 | − |
| $10^7$ | 0 | − | 0 | − | 24 | + | 48 | + | 48 | − | 12 | + |
| $10^8$ | 0 | − | 96 | + | 48 | + | 0 | − | 48 | + | 48 | − |
| $10^2$ *hardjo* | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − |
| $10^4$ | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − |
| $10^6$ | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − | 0 | − |
| $10^7$ | 24 | − | 0 | − | NT | + | 0 | − | 0 | − | 12 | − |
| $10^8$ | 0 | − | NT | + | 192 | + | 0 | − | 48 | − | NT | − |

* reciprocal of titre.

The characteristics of leptospiruria in mice infected with different serovars showed considerable variation. Leptospiruria in mice infected with *ballum* and *pomona* was observed for six months (at which time the experiment was terminated) whereas leptospiruria was not detected at any time in mice inoculated with *hardjo*. Only one urine sample from mice inoculated with *balcanica* was positive and this was taken on day 30 p.i. from a mouse inoculated with $10^8$ leptospires.

The prevalence of leptospiruria in groups of six mice infected with $10^8$ *ballum*, *balcanica* and *pomona* organisms and serially monitored for six months is given in Figure 15.2. Shedding was maintained in 100% of *ballum*-infected mice at all sampling times except for one mouse sampled on day 120 p.i. The prevalence of leptospiruria in groups of mice infected with *pomona* varied between 33% and 83% at different sampling times.

The time of onset and intensity of leptospiruria relative to serovar and infective dose is shown diagrammatically in Figure 15.3. The time of onset of leptospiruria in mice infected with both *ballum* and *pomona* was inversely related to the infective dose. All mice receiving more than $10^3$ *ballum* organisms were leptospiruric by day 11 p.i., whereas only 5 of 16 mice in the $10^3$, $10^2$ and $10^1$ groups (31%) were leptospiruric at this time. By day 14 p.i., all *ballum*-infected mice were leptospiruric. Only four mice that had been inoculated with *pomona* were leptospiruric on day 11 p.i. (9%) and these were in groups that had received $10^5$ to $10^8$ organisms. By day 14 p.i., leptospiruric mice were detected in all groups inoculated with *pomona*, although the prevalence of shedding within groups was considerably lower than was the case for *ballum*.

The intensity of shedding of *ballum* was high and was maintained throughout the experiment. The intensity of shedding of *pomona* was considerably lower (Figure 15.3.). *Ballum* organisms shed in urine were motile throughout the experiment. Motility was also maintained by *pomona*, however leptospires in 16% of urine samples collected from *pomona*-infected mice after day 60 p.i. were non-motile. The single urine sample from mice inoculated with *balcanica* that was positive contained one motile leptospire per ten dark-fields.

Figure 15.2. : Prevalence of leptospiruria in groups of six mice infected with *ballum*, *pomona* and *balcanica* at a dose rate of $10^8$ leptospires.

Inoculum

Days post-inoculation[+] and intensity of leptospiruria[++]

| 11[+] | 14 | 17 | 30 | 60 | 90 | 120 | 150 | 180 |
|---|---|---|---|---|---|---|---|---|
| 0 1[++] 2 3 4 | 0 1 2 3 4 | 0 1 2 3 4 | 0 1 2 3 4 | 0 1 2 3 4 | 0 1 2 3 4 | 0 1 2 3 4 | 0 1 2 3 4 | 0 1 2 3 4 |

ballum

$10^8$ $10^7$ $10^6$ $10^5$ $10^4$ $10^3$ $10^2$ $10^1$

pomona

$10^8$ $10^7$ $10^6$ $10^5$ $10^4$ $10^3$ $10^2$ $10^1$
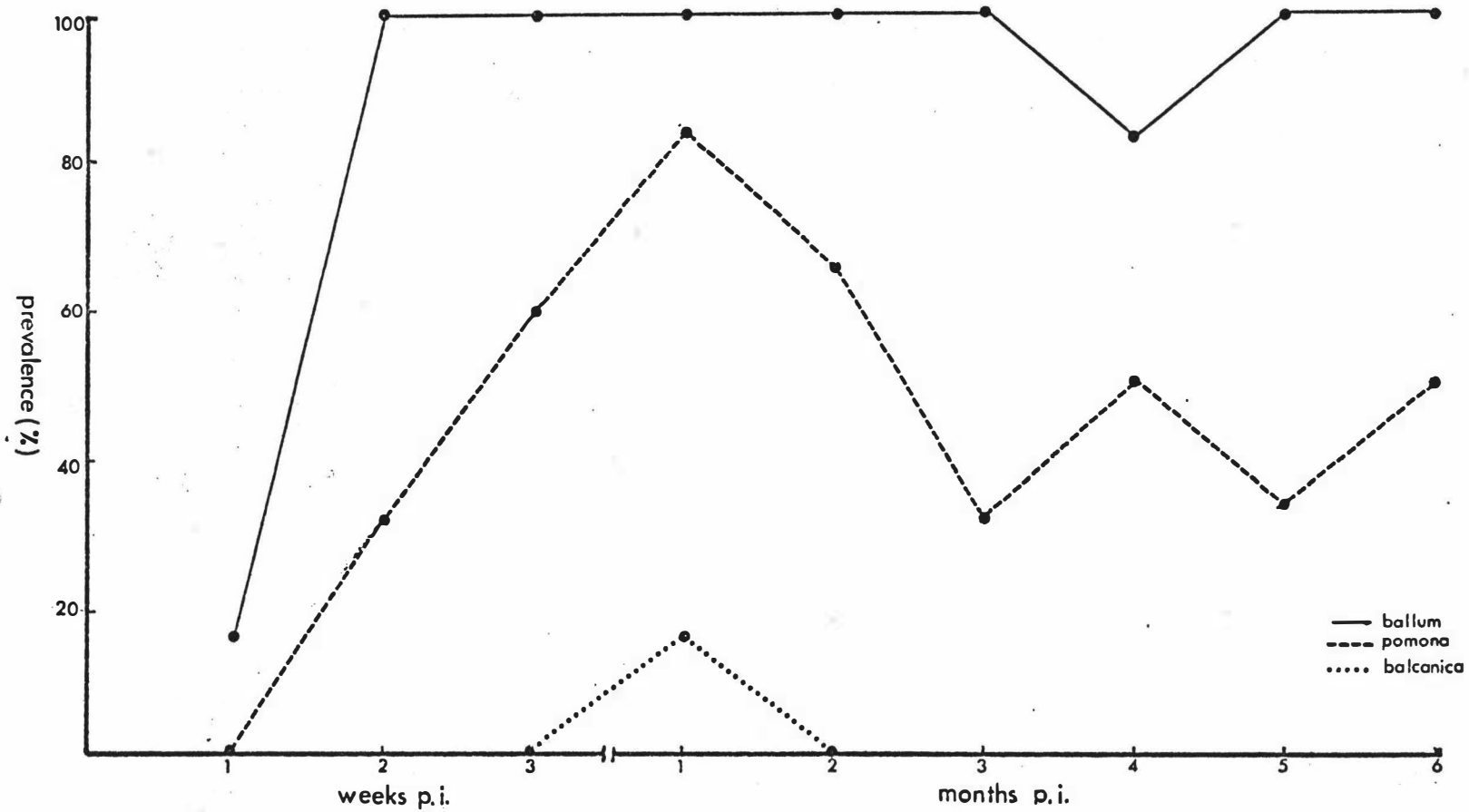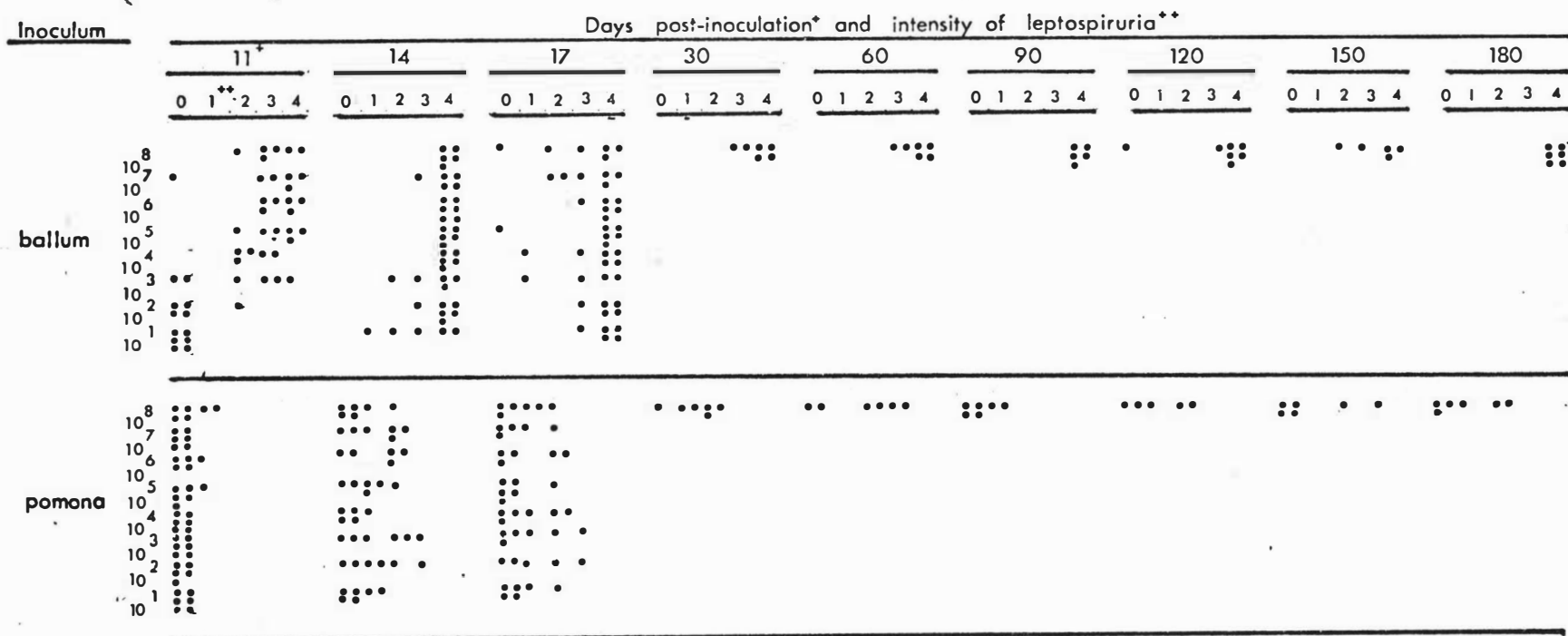
++ scoring system for intensity of leptospiruria is given in Table 15.1.

. dots represent individual mice in groups of six (at some sampling times, urine was obtained from only five of six mice in each group).

Figure 15.3. : Prevalence and intensity of leptospiruria at different times post-inoculation in groups of six mice infected with *ballum* and *pomona*.

All mice were bled and cultured at the termination of the
six-month experiment. Only one *ballum* titre (1:24) was detected
in sera from the six mice infected with *ballum*, however this organism
was recovered from all animals. Isolates were obtained from 4 of
6 mice that had been inoculated with *pomona* and titres of 1:96, 1:24
and 1:24 were present in three of these mice. *Balcanica* was isolated
from one of six mice that had been inoculated with this serovar and
this animal had a titre of 1:24. All mice in the group that had
been inoculated with *hardjo* were serologically and bacteriologically
negative. The serogroup of isolates recovered at the termination of
the experiment was confirmed by cross-agglutination.

A comparison of the sensitivity of dark-field microscopy relative
to kidney culture for the detection of short and long term infection
with *ballum*, *balcanica*, *hardjo* and *pomona* gave contrasting results.
Dark-field examination of urine on day 17 p.i. identified 41 of 45
mice that were infected with *ballum*. Thus the sensitivity of dark-
field microscopy relative to culture was 91%. All mice from which
*ballum* was isolated on day 180 p.i. were positive by dark-field
microscopy (100% sensitivity). These dark-field microscopy reults
reflected the high intensity and constancy of shedding of *ballum*.

Dark-field microscopy was considerably less sensitive for the
identification of mice infected with *pomona*. The range in sensitivity
for different groups of mice at day 17 p.i. was 17 to 80%. The
sensitivity of dark-field microscopy for detecting long-term *pomona*
infection at 180 days p.i. was 75%. This lower sensitivity reflected
the lower intensity of shedding of *pomona* than *ballum*.

The sensitivity of dark-field microscopy for detecting leptospiral
infection in mice infected with *balcanica* and *hardjo* was 0. This did
not rule out the possibility that mice infected with these serovars
may have had a low intensity, transient leptospiruria, however it was
apparent that there were significant differences in leptospiruria
in mice infected with *ballum* and *pomona* compared with *balcanica* and
*hardjo*.

Experiment 3 : Infectivity of leptospires shed in mouse urine and natural transmission of *ballum, balcanica, hardjo* and *pomona*.

Intraperitoneal incculation of urine from mice that had been inoculated with the above-mentioned serovars 21 days previously resulted in transfer of infection of *ballum* and *pomona* but not *balcanica* and *hardjo*.    All the mice inoculated with mouse urine containing *ballum* or *pomona* were culture-positive at day 21 p.i. Dark-field examination of urine inocula from mice that had been previously inoculated with *balcanica* and *hardjo* failed to demonstrate the presence of leptospires.  *Balcanica* was cultured from one urine inoculum; urine inocula from *hardjo*-inoculated mice were negative.

Natural transmission of leptospiral infection between mice was demonstrated with *ballum*.  The three males caged with *ballum*-infected females were found to be leptospiruric by dark-field micro-scopy four weeks after introduction and *ballum* was isolated from all kidney cultures.  *Pomona*  was not transmitted to the three males introduced to infected females.  All dark-field examinations of urine and kidney cultures from male mice were negative.  Female mice were found to be shedding *pomona* over the duration of this experiment and *pomona* was cultured from two of the three females at the termin-ation of the experiment.  There was no evidence of infection in males that were introduced to females that had been inoculated with *balcanica* and *hardjo*.  *Balcanica*  was recovered at the termination of the experiment from one of the three females inoculated with this serovar.  Females that had been inoculated with *hardjo* were negative on kidney culture.

Experiment 4 : Age susceptibility to infection with *ballum, balcanica, hardjo* and *pomona*.

The results of this experiment are given in Table 15.3.  No difference in age susceptibility was shown with *ballum* or *balcanica*. A small difference in age susceptibility was shown with *pomona*. Weanling mice were infected with $10^1$ organisms, however adults were insusceptible to infection by this inoculum.  Only one of the adult mice inoculated with $10^2$ *pomona*  organisms became infected whereas all weanlings were infected at this dose rate.  Neither weanlings nor

Table 15.3 : Age susceptibility of mice to infection with *ballum, pomona, balcanica* and *hardjo*.

| No. orgs. in inoculation | *ballum* | | *pomona* | | *balcanica* | | *hardjo* | |
|---|---|---|---|---|---|---|---|---|
| | weanling | adult | weanling | adult | weanling | adult | weanling | adult |
| $10^8$ | +++ * | +++ | +++ | +++ | +-- | +-- | --- | --- |
| $10^6$ | +++ | +++ | +++ | +++ | --- | +-- | --- | --- |
| $10^4$ | +++ | +++ | +++ | ++- | --- | --- | --- | --- |
| $10^3$ | +++ | ++- | ++- | +++ | --- | --- | --- | --- |
| $10^2$ | +++ | ++- | +++ | +-- | NT | NT | NT | NT |
| $10^1$ | +++ | ++- | +++ | --- | NT | NT | NT | NT |

* 3 mice in each gp. + = culture positive

‑ = culture negative.

adults were susceptible to *hardjo* in this experiment. This *hardjo* culture also failed to infect hamsters. Mean death times of hamsters infected with $10^8$ organisms of the *ballum*, *balcanica* and *pomona* cultures used in this experiment were 5.0, 13.3 and 4.7 days respectively.

Leptospires were cultured back from all dilutions of cultures used in this experiment. Control groups of mice were negative.

Experiment 5 : Investigation of difference in the age prevalence of infection.

Four of five two-week old mice from a *ballum*-infected dam were found to be resistant to challenge with *ballum* (80%). One mouse was positive by kidney culture 21 days p.i. Similarly, 4 of 5 four week old mice were resistant to challenge (80%). *Ballum* was recovered from 4 of 5 mice that had been challenged at eight weeks of age (80%). All littermates maintained as controls were negative on culture. Thus there was no natural tranmission from the dams to their offspring, despite the fact that large numbers of leptospires were shed in urine.

The results of the experiment to investigate sexual tranmission of *ballum* in adult mice are given in Table 15.4. Infection was transmitted in all groups of mice. All males paired with infected females became infected and 4 of 5 females paired with infected males became infected (80%). Similarly, *ballum* was transmitted between all pairs of infected and non-infected males and 4 of 5 pairs of infected and non-infected females (80%). Thus transmission was not limited to venereal transmission. Control pairs of mice were negative.

Figure 15.4 describes the pattern of transmission of *ballum* within litters of mice in which two-week old males were inoculated with *ballum*. Leptospiruria was first observed in infected males six days after inoculation. All inoculated males were leptospiruric by day 12 p.i. No infection was detected in female litter-mates until they were 39 days of age, when urine from one female in the litter was positive. By 50 days of age, infection had been

Table 15.4 : Transmission of *ballum* infection between different-
sex and same-sex pairs of laboratory mice.

| Pair No. | Infected | Non-infected | Transmission |
|---|---|---|---|
| 1 | F * | M * | + |
| 2 | F | M | + |
| 3 | F | M | + |
| 4 | F | M | + |
| 5 | F | M | + |
| | | | |
| 1 | M | F | + |
| 2 | M | F | − |
| 3 | M | F | + |
| 4 | M | F | + |
| 5 | M | F | + |
| | | | |
| 1 | M | M | + |
| 2 | M | M | + |
| 3 | M | M | + |
| 4 | M | M | + |
| 5 | M | M | + |
| | | | |
| 1 | F | F | − |
| 2 | F | F | + |
| 3 | F | F | + |
| 4 | F | F | + |
| 5 | F | F | + |

F = female

M = male

transmitted to both dams and by 60 days of age, all litter-mates were infected. Thus transmission of *ballum* did not occur until infected mice had reached sexual maturity.

The same experiment was repeated using *pomona*-infected, two-week old males and the results are presented diagrammatically in Figure 15.5. Leptospiruria was first detected in male mice at 12 days p.i. By day 25 p.i. *pomona* was being shed by all inoculated mice. The only transmission of this infection was to one female littermate who was positive on kidney culture at the termination of the experiment. Cultures of other littermates and dams were negative.

Experiment 6 : Ratios of serological and bacteriological prevalence.

These ratios varied considerably for different serovars (Table 15.5.). Ratios calculated at day 17 p.i. and day 180 p.i. were unity or less for *ballum* and *pomona*. The ratios at day 17 p.i. for *balcanica* and *hardjo* were considerably higher than unity (2.7 :1 and 4.3 :1 respectively). Only one mouse was still infected with *balcanica* at day 180 p.i. The ratio of serological to bacteriological prevalence of *balcanica* infection in this group at this time was 1:1.

DISCUSSION

Serial passage demonstrated that *ballum*, *balcanica* and *pomona* were highly virulent for hamsters. The small variations in death times during the serial passage of these serovars was probably due to variation in the number of leptospires in the inocula of kidney homogenate rather than changes in the virulence of the infecting serovar. Baker and Baker (1970) demonstrated an inverse relationship between the log infective dose of leptospires and death time in hamsters. Similar findings were reported by Ellinghausen and Painter (1976).

*Hardjo* was of low infectivity for hamsters. Infection was demonstrated in only a small proportion of inoculated hamsters and no clinical symptoms were seen in these animals.

Figure 15.4. : Transmission of *ballum* within family groups of laboratory mice.
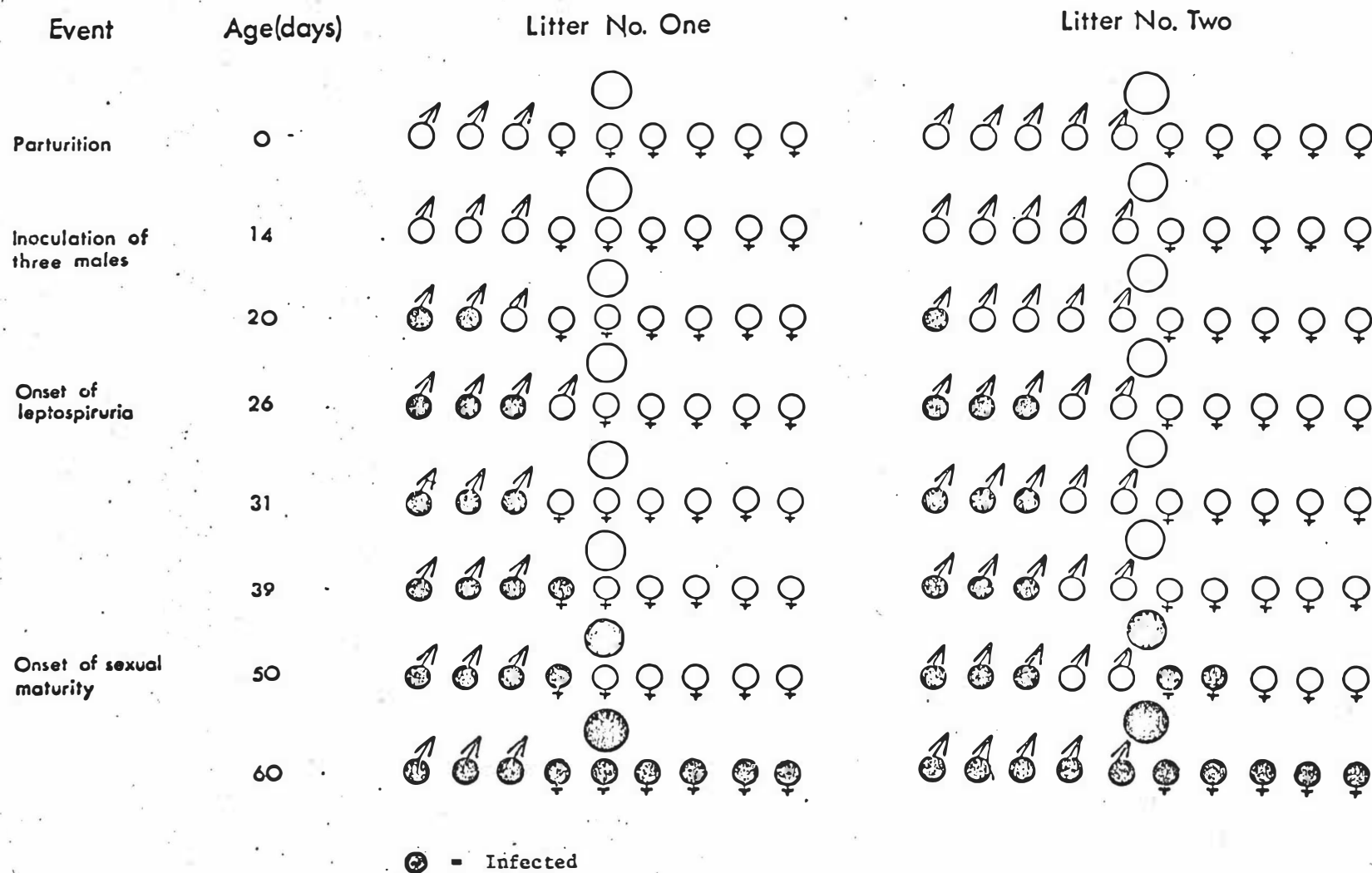
⊙ = Infected

Figure 15.5. : Transmission of *pomona* within family groups of laboratory mice.
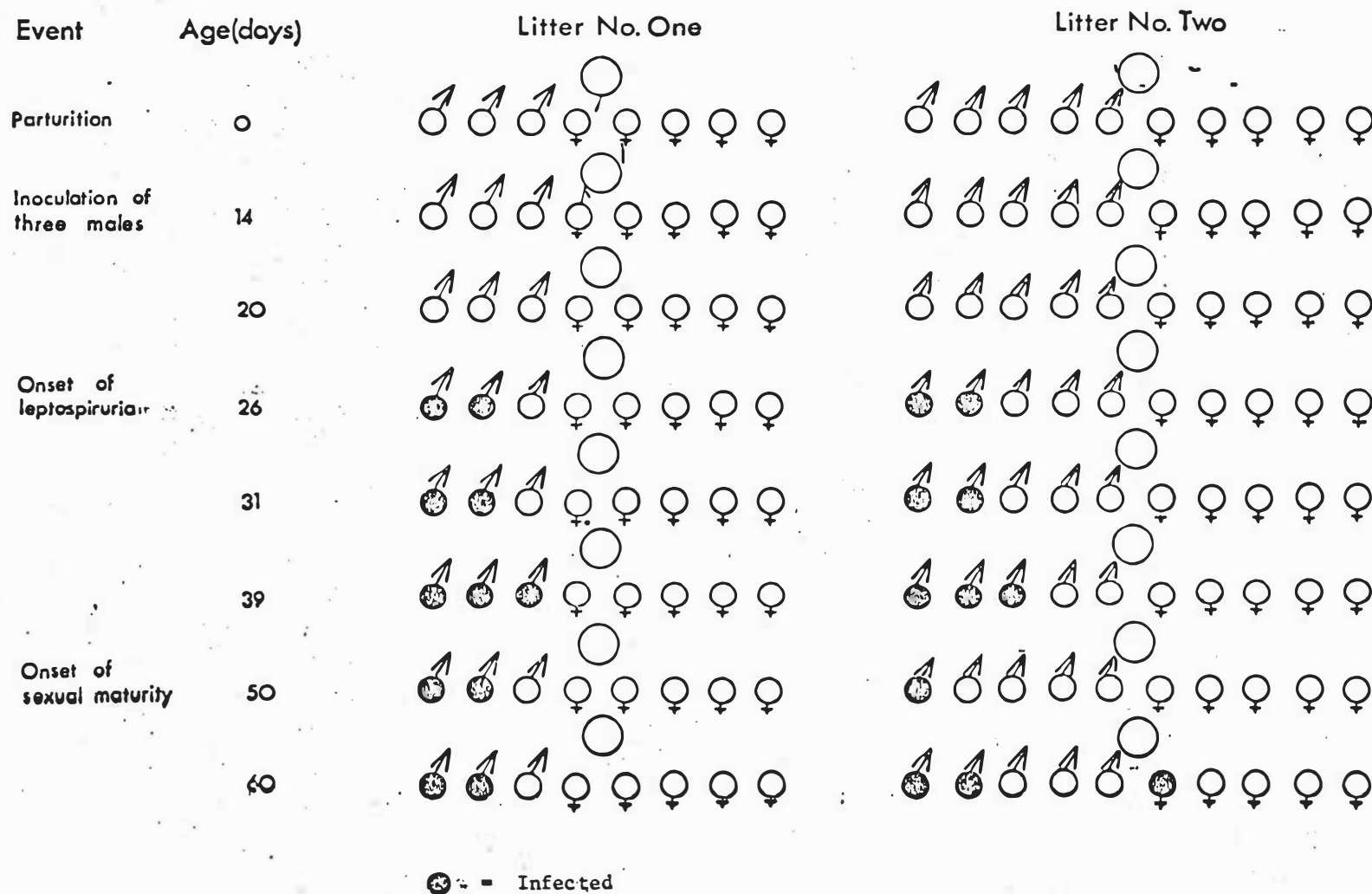
Table 15.5 : Serological and bacteriological prevalence of experimental *ballum*, *balcanica*, *hardjo* and *pomona* infection in mice.

| Serovar | Day 17 p.i. | | | Day 180 p.i. | | |
|---------|-----------|------------|-------|-----------|------------|-------|
|         | sero prev. | bact. prev. | ratio | sero prev. | bact.prev. | ratio |
| *ballum* | 44 | 100 | 0.4:1 | 17 | 100 | 0.2:1 |
| *balcanica* | 35 | 13 | 2.7:1 | 17 | 17 | 1:1 |
| *hardjo* | 13 | 3 | 4.3:1 | 0 | 0 | 0 |
| *pomona* | 92 | 92 | 1:1 | 50 | 67 | 0.8:1 |

There was a marked difference in the response of laboratory mice to infection with *ballum, balcanica, hardjo* and *pomona,* and this species provided an ideal model to evaluate the criteria used by Emanuel (1959), Roth *et al* (1963) and Chernukcha *et al* (1974) to define a maintenance host for a particular serovar. These criteria were the (i) clinical response to infection, (ii) characteristics of leptospiruria, (iii) serological response to infection, (iv) serological and bacteriological prevalence of infection and the ratio of these parameters and (v) the age susceptibility to infection. Additional criteria introduced in this study were (vi) infective dose and (vii) demonstration of natural transmission.

## (i) Clinical response to infection

No clinical symptoms were observed in mice infected with *ballum, balcanica, hardjo* or *pomona* and therefore infection with these serovars could not be differentiated by this criterion. Laboratory mice are generally insusceptible to clinical leptospirosis (Stalheim, 1966; Birnbaum *et al,*1972). The presence of unsuspected endemic *ballum* infection in a laboratory mouse unit is further evidence of the lack of a clinical response to infection. It has been reported that a few serovars, e.g. *australia B*, are highly pathogenic for mice (Emanuel, 1959; Faine, 1962).

## (ii) Characteristics of leptospiruria

A detailed investigation of the characteristics of leptospiruria in mice infected with different serovars revealed wide variations Roth *et al* (1963) defined leptospiruria in a maintenance host as being the long-term excretion of large numbers of viable organisms. Chernukcha *et al* (1974) considered constancy and a high prevalence of leptospiruria in an infected population to be additional charact-eristics.

The serovar which most adequately satisfied these criteria was *ballum.* High intensity leptospiruria was constant in all mice for the six month duration of the experiment. Leptospiruria in mice infected with *pomona* was of lower intensity and showed some inter-

mittency, however, 50% of mice were still shedding organisms six
months after infection. Thus leptospiruria in mice infected with
*pomona* was also characteristic of that in a maintenance host.

Only one urine sample from mice infected with *balcanica* was
positive by dark-field microscopy and no urine samples from mice
infected with *hardjo* were positive. Leptospiruria in mice infected
with these two serovars was therefore not characteristic of that
of a maintenance host.

(iii) Serological response to infection

There was a considerable difference in the serological response
of mice infected with different serovars. *Ballum* titres were very
low with a GMT of 1:29 whereas *pomona* titres had a much higher GMT
of 1:285. *Balcanica* and *hardjo* titres had GMT's of an intermediate
level (1:54 and 1:81 respectively). Sera from chronically-infected
animals also demonstrated differences in the persistence of titres.
Sporadic low titres to *ballum*, *balcanica* and *pomona* were present six
months after inoculation, whereas *hardjo* titres were not detectable
at this time.

Chernukcha *et al* (1974) considered that a characteristic of
leptospiral infection in a maintenance host was a high and persistent
titre and infection in an accidental host was characterised by a low
and transient titre. Application of these serological criteria to
laboratory mice infected with different serovars in the present study
indicated that mice were maintenance hosts only for *pomona*.

(iv) Serological and bacteriological prevalence of infection

Kidney cultures revealed that different serovars had different
abilities to infect, and persist in, laboratory mice. Bacteriological
criteria established that laboratory mice were maintenance hosts for
*ballum* and *pomona* and accidental hosts for *hardjo*. Infection with
*balcanica* was not clearly defined by bacteriological criteria (one
of six *balcanica*-infected mice was still positive six months after
inoculation).

Roth *et al* (1963) suggested that the ratio of serological and bacteriological prevalence should be considered when defining leptospiral infection in a maintenance host. A maintenance host would be expected to have "a high bacteriologic rate of infection not exceeded greatly by the serologic rate". An accidental host for a particular serovar would have " a high serologic rate of infection many times greater than the bacteriologic rate of infection". A consideration of the serological and bacteriological prevalences from the laboratory mouse model indicated that this species was a maintenance host for *ballum, balcanica* and *pomona*. The high ratio of serological to bacteriological prevalence for mice inoculated with *hardjo* (4.3:1) indicated that this species was an accidental host for this serovar.

## (v) Age susceptibility to infection

Chernukcha *et al*(1974) considered that there was no difference in age susceptibility to infection with a particular serovar in a maintenance host, whereas juveniles of an accidental host species were much more susceptible than adults. An investigation of this criterion for differentiating maintenance and accidental hosts gave inconclusive results in the present study. Juvenile mice inoculated with *balcanica* and *hardjo* were not more susceptible than adults to infection, yet previous criteria had indicated that laboratory mice were accidental hosts for these serovars. There was a small difference in age susceptibility of juvenile and adult mice inoculated with *pomona*, however this was only recognised at an infective dose of ten organisms.

## (vi) Infective dose

An additional criterion used in the present study to define infection in a maintenance host was the infective dose of a serovar. There was a marked difference in the minimum infective dose (MID) of different serovars. Infection was established in all mice inoculated with from $10^1$ to $10^8$ *ballum* organisms. The very low MID and consistent nature of the prevalence of infection in each treatment group, indicated that the mouse was a good maintenance host for *ballum*. The MID for mice infected with *pomona* was also $10^1$ organisms, although the prevalence of infection in the groups receiving $10^1$ and $10^2$ organisms was lower than for *ballum*. Therefore a consideration of MID

indicated that laboratory mice were maintenance hosts for *pomona*.
The MID of *balcanica* and *hardjo* was very high ($10^7$ organisms) and
the prevalence of infection in groups receiving $10^7$ and $10^8$ organisms
was considerably lower than was the case for *ballum* and *pomona*.
Thus the MID of *balcanica* and *hardjo* indicated that the laboratory
mouse was an accidental host for these serovars.

A summary of the application of the serological and bacteriological
criteria that were considered indicative of leptospiral infection in a
maintenance host is given in Table 15.6.   The maintenance host
criteria of Roth *et al*(1963) were satisfied in the laboratory mouse
for *ballum* and *pomona*.    The laboratory mouse was shown to be an
accidental host for *hardjo* but was not clearly defined as either
a maintenance or accidental host for *balcanica*.

The serological response and age susceptibility criteria
used, in addition to leptospiruria, by Chernukcha *et al,*(1975)
gave contradictory results and it is considered that these criteria
of leptospiral infection in a maintenance host are ill-defined.  In
particular, it has been well established that both laboratory and
free-living house mice are maintenance hosts for *ballum*, yet the
serological response criterion of Chernukcha *et al* (1974) did not
support this.  The age susceptibility criterion also indicated that
laboratory mice were maintenance hosts for *hardjo* and *balcanica,*
yet this was not justified by other criteria characterising leptospiral
infection in a maintenance host.

A consideration of the MID of a serovar and the serological and
bacteriological criteria of Roth *et al* (1963) indicated that the mouse
was a maintenance host for *ballum* and *pomona*  and an accidental host
for *balcanica* and *hardjo*.  Results of field investigations, both in
New Zealand and in other countries, support these findings, in respect
of *ballum, balcanica* and *hardjo*  (Babudieri, 1958; Clark, 1961;
Parnas *et al*, 1961; Roth, 1970; Fennestad and Borg-Petersen,1972).
The application of the previously described serological and bacterio-
logical criteria characterising infection in a maintenance host did
not support field data in respect of *pomona*.

Table 15.6 : Application of the serological and bacteriological criteria defining a maintenance
host for a serovar to experimental infection with *ballum*, *balcanica*, *hardjo* and *pomona*.

Criterion defining a maintenance host

| Serovar | Roth *et al*, 1963 | | Chernukcha *et al*, 1974 | | Infective Dose |
|---|---|---|---|---|---|
| | leptospiruria | ratio sero:bact.prevalence | immune response to infection | age susceptibility | |
| *ballum* | + | + | - | + | + |
| *balcanica* | - | + - | - | + | - |
| *hardjo* | - | - | - | + | - |
| *pomona* | + | + | + | + | + |

(vii)   Demonstration of natural transmission

The maintenance of an endemic focus of leptospiral infection
in a particular host species is based on intra-species transmission.
Therefore, the demonstration of intra-species transmission was
introduced in this study as a further criterion of leptospiral
infection in a maintenance host.  Artificial intra-species
transmission by intraperitoneal inoculation of urine was demonstrated
with *ballum* and *pomona* but not  *balcanica* and *hardjo*.  This demon-
strated that *ballum* and *pomona* leptospires shed by mice were infective
for their own species by artificial inoculation.  *Balcanica* shed in
mouse urine was not infective by artificial inoculation.  This was
probably a reflection of the low numbers of leptospires present in
the urine of mice infected with *balcanica* and the high MID ($10^7$
organisms) needed to infect laboratory mice.

The demonstration of natural intra-species transmission between
adult mice could be achieved only with *ballum*.  Transmission to exposed
mice occurred in all cases and this reinforced the designation of
the laboratory mouse as a maintenance host for *ballum*.  All in-contact
adult males exposed to females infected with *pomona* and *balcanica*
were bacteriologically negative on kidney culture.  ( No infection
was demonstrated in females inoculated with *hardjo* or in-contact
males.)  Natural intra-species transmission of *ballum* was also
demonstrated within family groups.  Infected males transmitted this
infection to all littermates of both sexes and also to the dams.
Natural transmission from *pomona*-infected males to littermates occurred
in only 1 of 12 animals (8%) and there was no transmission to dams.
The criterion of demonstration of natural transmission therefore
defined the laboratory mouse as a maintenance host for *ballum* and an
accidental host for *pomona*.   This differentiation is supported by
field data but was unable to be demonstrated using the previously
described serological and bacteriological criteria.

Artificial transmission with *pomona* was demonstrated in the
laboratory mouse model but despite the very low MID of *pomona* by
intraperitoneal inoculation, natural tranmission did not occur
(except in one seven-week old mouse).  This observation suggests
that it is host factors that ultimately determine if an animal

species is a maintenance host for a particular serovar. Without natural transmission of a serovar, an endemic focus cannot be maintained.

The concept of maintenance hosts for particular serovars in a specific ecosystem is a complex one. As pointed out by Turner (1967  ), one host species may act as a maintenance host for more than one serovar and a particular serovar may have maintenance hosts of different species in different regions. In addition, the maintenance host for one serovar may be an accidental host for other serovars and two or more maintenance hosts for the same serovar may be found in the same ecosystem.

In light of the results obtained from the study of leptospirosis in both field populations and the laboratory mouse model, both maintenance hosts and maintenance populations have been re-defined in the following manner :

1.  A maintenance host is defined as " an animal which is capable of acting as a natural source of leptospiral infection for its own species."

Whether an animal is capable of acting as a maintenance host for a specific serovar depends on host genotype. Leptospiral infection in a maintenance host is characterised by :-

(a) high susceptibility of the host to infection i.e. low infective dose.
(b) low pathogenicity of the serovar for the host.
(c) long-term kidney infection relative to the systemic phase of infection.
(d) natural transmission within species.

2.  A maintenance population is defined as " a population of a species of animal which acts as a continuous reservoir of a serovar in a specific ecosystem."

A maintenance population is characterised by :-

(a) natural tranmission of infection between individuals

of the same generation within the population.

(b) natural transmission between successive generations of the population.

3.  The establishment and prevalence of leptospiral infection in a maintenance population is dependant on :-

(a) initial exposure to the infectious agent.

(b) the population dynamics operating within the population.

(c) population behaviour.

(d) environmental conditions and, in the case of domestic animals, husbandry procedures.

(e) concurrent disease and physiological status of the population.

(f) the stability of the leptospiral serovar involved.

4.  If unnatural conditions are operating, it is possible that a maintenance population may occur that is made up of individuals with atypical attributes of a maintenance host for the serovar involved. This situation would not continue indefinitely.

The laboratory mouse model was also used to investigate difference in the age prevalence of infection which often occurs with infection with a particular serovar in a maintenance host. Maternally-derived antibody played an important role in protecting juvenile mice from infection by experimental inoculation. This protection was evident at 14 and 28 days post-partum but not at 56 days. Other workers have reported that passive immunity provided by maternally-derived antibodies persists in laboratory mice for at least 30 days (Kemenes and Szeky, 1966) and 51 days (Stoenner *et al*, 1958). In the present study, passively derived protection was not absolute, as 1 of 5 neonates that were challenged (20%) became infected. It would appear that such an occurrence is rare under natural conditions however, as Stoenner *et al* (1958) and Kemenes and Szeky (1966) did not identify any juvenile carriers in laboratory mouse colonies endemically-infected with *ballum* and *sejroe*. In an endemically-infected mouse population, the passive acquisition of maternal antibody is therefore associated with an absence of infection in mice of less than 30 to 50 days of age. It

is during this period that laboratory mice (and probably free-living populations of house mice) reach sexual maturity (Thieler, 1972).

*Ballum* infection was transmitted between all single sex and different sex pairs of laboratory mice. Thus transmission was not dependant on coitus but it may have been dependant on behaviour patterns associated with sexual maturity. This was confirmed by Experiment 5. Transmission to dams and littermates occurred only after the period of onset of sexual maturity of infected mice had been reached. An age difference in the prevalence of infection in laboratory mice was therefore associated with the onset of sexual maturity. Similar observations have been made in field studies of several free-living species (see previous chapters). It has been suggested that such age differences in the prevalence of infection in maintenance hosts is due to venereal transmission of the particular serovar. Until transmission in these species is shown to be exclusively venereal, it can only be said to be associated with the onset of sexual maturity. Although transmission may be dependant on behaviour changes following sexual maturity, it has been shown in this study that it is not dependent on coitus.

The waning of maternally-derived protection and the onset of sexual maturity in laboratory mice occurs at approximately the same time. Experimental results in mice with no maternally-derived antibody have indicated that it was the onset of sexual maturity that allowed natural transmission of *ballum* to occur. The importance of passively-derived antibody in preventing infection in neonates can only be investigated by exposing neonates from non-infected dams to leptospiruric dams. This was not attempted in the present study. In animals where sexual maturity occurs much later than the waning of passively-derived maternal antibodies, e.g. in the possum, the dependance of differences in the age-specific prevalence of infection on sexual maturity is more easily demonstrated (see Chapter VIII).

Birnbaum *et al*(1974) put forward the hypothesis that there was a cyclic occurrence of endemic infection with *grippotyphosa* in free-living house mice in Israel. This was based on experimental studies that indicated that the offspring of infected dams with high titres

were immune to challenge for at least 450 days, i.e. the effective life-span of house mice. This was not the case in laboratory mice infected with *ballum*, as progeny of recently-infected dams were susceptible to infection at eight weeks of age. Similarly, long-term passive protection was not found in laboratory mouse colonies infected with *sejroe* in Europe (Kemenes and Szeky,1966).

The results of experimental infections with *ballum, balcanica, hardjo* and *pomona* in the laboratory mouse model support field data from free living *Mus musculus* populations in New Zealand and other countries (Chapters XIII and XIV). Extrapolation of the experimental findings to free-living house mice populations indicate that endemic infection with serovars other than *ballum* would not occur. Because of the low MID of *pomona* for laboratory mice, it is possible that sporadic *pomona* infections occur in free-living populations. Evidence of this was found at the Massey Pig Research Centre and Number 1 Dairy Farm (Chapter XIV). The laboratory mouse model indicated that this infection would not be transmitted from mouse to mouse and therefore an endemic focus would not become established in this species.

SUMMARY AND CONCLUSIONS

1. Laboratory mice were used as an experimental model to define the characteristics of leptospiral infection with a particular serovar in a maintenance host.

2. A maintenance host is defined as "an animal which is capable of acting as a natural source of leptospiral infection for its own species".

3. Leptospiral infection in a maintenance host is characterised by
   (a) high susceptibility of the host to infection i.e.low MID.
   (b) low pathogenicity of the serovar for the host.
   (c) long-term kidney infection relative to the systemic phase of infection.
   (d) natural transmission within a species.

4. A maintenance populations is defined as " a population of a species of animal which acts as a continuous reservoir of a serovar in a specific ecosystem". This is characterised by natural transmission of infection between individuals of the same and successive generations in the population.

5. Passively-derived maternal antibodies protected juvenile mice from experimental challenge with *ballum* for up to eight weeks.

6. An age difference in the prevalence of *ballum* infection in laboratory mice was associated with the onset of sexual maturity. Transmission of infection was not proven to be exclusively venereal but was more broadly considered to be due to a change in behavioural patterns that followed sexual maturity.

7. Extrapolation of the results of the experimental study indicates that free-living house mouse populations may act as maintenance hosts for *ballum*,but only accidental hosts for *pomona, balcanica* and *hardjo*.

8. Results of the experimental study were supported by field data presented in Chapters XIII and XIV.

CHAPTER XVI

GENERAL DISCUSSION

Prior to 1975, there was little information available on the prevalence of leptospiral infection in free-living animals in New Zealand, despite the widespread recognition of high rates of infection in domestic stock and an important public health problem in man. Concurrent investigations by Brockie (1975; 1977) and Brockie and Till (1977) and those detailed in this thesis have established that leptospirosis is widespread in wildlife in this country.

The investigations reported in this thesis can be divided into four main areas: surveys to establish the prevalence of leptospiral infection in free-living animals in New Zealand, immunological and bacteriological studies, experimental infections and epidemiological investigations. The results of these investigations have led to a wider understanding of the epidemiology of leptospirosis in free-living animals in New Zealand and a redefinition of the characteristics of a maintenance host for a particular serovar.

Hebdomadis serogroup titres were found in high prevalence in sera from possum populations inhabiting farmland in the southern half of the North Island of New Zealand. A cultural survey resulted in 57 isolates being obtained from 154 animals (37%) and eight of these were typed by cross-agglutination absorption as serovar *balcanica*. No evidence of *hardjo* infection was found in pastoral possum populations. Infection with *hardjo* and *balcanica* cannot be distinguished serologically and it is considered that previous reports of endemic *hardjo* infection in possums in New Zealand (Brockie, 1975; de Lisle *et al*, 1975) are open to question.

The epidemiology of *balcanica* infection in possum populations was the subject of detailed investigation. Such epidemiological investigations of disease in free-living populations are subject to problems not experienced in investigations of domestic animals, one of the most important of which is the limitations of cross-sectional sampling. Experimental investigations are therefore an important adjunct to field

studies. Results must also be interpreted relative to a knowledge of the biology and ecology of the species being investigated.

Possums were shown to be typical maintenance hosts for *balcanica*. The high prevalence of titres in possum populations was similar to the bacteriological prevalence and this indicated that the possum was a long-term carrier of this serovar. Experimental investigations demonstrated that 50% of possums were still shedding leptospires one year after infection. Infection was easily established and asymptomatic. It is probable that following infection, titres persist in possums for considerably longer than a year. The high prevalence of titres in adult possums was reflected in the high prevalence of passively-derived antibody titres in sera from pouched-young and juvenile possums, 40% of which were still sero-positive at six months of age. Chromatographic studies demonstrated that these passively-derived antibodies were of the IgG class. Following the loss of passively-derived protection, juveniles were found to be fully susceptible to experimental challenge, however, similar free-living animals remained uninfected until sexually mature.

The restriction of *balcanica* infection in possums to sexually mature animals was independant of population density or environment. A similar age-differentiation of leptospiral infection has been reported in other free-living species. It is hypothesised that this finding reflects an important adaption of leptospires to their maintaining hosts that ensures the continued transmission and therefore survival of the organism. Evidence is presented that suggests that transmission of *balcanica* is dependant on direct contact between animals rather than indirect transmission via the environment. The type of contact required is thought to be associated with behavioural changes that follow the onset of sexual maturity. With the perpetuation of a species being dependant on reproduction, so the perpetuation of a particular leptospiral serovar, an obligate parasite, is ensured. If transmission within a maintenance host species was dependant on the environment, the maintenance of an endemic focus of leptospirosis would be dependant on environmental factors favouring the survival of leptospires. Such a dependance would represent a much less efficient parasitic adaption than that described.

A consistent paradoxical reaction to *hardjo* was found in sera from possums infected with *balcanica*. This phenomenon was not found in sera from other species infected with this serovar, although equivalent cross-reactions between *hardjo* and *balcanica* agglutinins occurred. Chromatographic studies of possum sera revealed that this paradoxical agglutination reaction was attributable exclusively to antibodies of the IgM class. The immunological basis for this finding remains unknown and suggests an area for further investigation. Other workers have reported that *balcanica* is "antigenically poorer" than other serovars of the Hebdomadis serogroup (Manev and Siromashkova, 1970), however, there have been no other immunological investigations of infections caused by this serovar. Chromatographic studies also indicated that the antibody response of possums to *balcanica* infection was somewhat dissimilar to that occurring in leptospiral infection with other serovars in eutherian mammals. The transition of agglutinating activity in sera from antibodies of the IgM class to antibodies of the IgG class was considerably slower with increasing time after initial infection than that occurring in eutherian mammals. Significant proportions of IgM were still present in sera one year after inoculation. These differences in the immunological response to leptospiral infection in possums, compared with eutherian mammals, may be a reflection of the different phyletic line of marsupials.

The occurrence of a high prevalence of endemic *balcanica* infection in possums must be considered in the epidemiology of Hebdomadis serogroup infections in domestic animals and man in New Zealand. A central theme of the investigation detailed in this thesis is that of the remarkable host-parasite adaption displayed by particular serovars for their host species. Only two serovars of the Hebdomadis serogroup are known to be present in New Zealand and well-defined maintenance host-parasite relationships have been demonstrated for *hardjo* and cattle, and *balcanica* and possums. It is evident however, that although the continued presence of a particular serovar in an ecosystem depends on continued transmission within a maintenance host species, individuals of other species may become infected when they intrude on the habitat of a maintenance host population or a maintenance host intrudes on the habitat of other species. It is therefore likely that sporadic, unsuspected *balcanica* infection occurs in domestic stock and man in New Zealand.

Hebdomadis serogroup titres, all attributed to *hardjo*, have been found in cattle, horses, sheep and humans. Experimental studies in sheep (unpublished) and cattle (Hellstrom, 1978) have demonstrated that infection with *balcanica* and *hardjo* cannot be distinguished serologically. The isolation of *balcanica* from man, cattle and pigs in Eastern Europe supports the contention that this serovar must be considered when investigating the epidemiology of Hebdomadis serogroup infections in domestic stock and man.

Shifts in the natural nidality of leptospirosis may occur within specific ecosystems (Shenberg *et al*, 1977). The bacteriological surveillance of Hebdomadis serogroup infection in cattle, sheep and horses in New Zealand should therefore be an important consideration. In this respect, the use of the *in vitro* haemolysin test described in Chapter X for the differentiation of *balcanica* and *hardjo* isolates would be of considerable benefit. It has been reported by Hellstrom (1978) that the "control and elimination of bovine *hardjo* infection within a closed population of cattle may be a practical possibility". If such measures are implemented in the future, the presence of endemic *balcanica* infection in possums may constitute a risk to *hardjo*-free bovine populations. At present, the high prevalence of endemic *hardjo* infection in bovines may prevent significant levels of infection with *balcanica*. Protection against the haemolytic effect of *balcanica* for red blood cells from ruminants infected with *hardjo* was demonstrated in Chapter X. The removal of endemic bovine *hardjo* infection and the close ecological association of possums and cattle could allow the introduction of *balcanica*, with significant economic and public health consequences.

Investigations of free-living populations of small mammals other than possums revealed that Ballum serogroup infection was endemic in ship rats, house mice and hedgehogs. Serological surveys at low minimum serum dilutions revealed that the prevalence of *ballum* titres in these populations was low, however, high bacteriological prevalences were revealed. These species are all considered to be maintenance hosts for leptospires of the Ballum serogroup in New Zealand. Single isolates from all species were identified by cross-agglutination absorption as serovar *ballum*.

A consideration of the biology and ecology of ship rats, house mice and hedgehogs in New Zealand indicated that leptospires of the Ballum serogroup were maintained independantly by the different species in both natural and syanthropic foci. The differentiation in the age-specific prevalence of leptospiral infection found in possums was also found in these species and it is suggested that direct contact between animals is of more importance in the maintenance of endemic Ballum serogroup infection in ship rats, house mice and hedgehogs than indirect transmission of leptospires via the environment.

The occurrence of Norway rat populations of different population densities at different suburban and country rubbish tips provided an opportunity to investigate Ballum serogroup infection in this species, a rodent not usually infected with these organisms. The prevalence of infection in different populations was significantly correlated with population density, with 43% of Norway rats from a high density population being bacteriologically positive. Epidemiological studies in other countries and those described in this study suggest that the Norway rat is not a true maintenance host for leptospires of the Ballum serogroup. It is apparent, however, that a focus of infection can be maintained in biotopes where large numbers of rats are present.

Investigations of free-living carnivores and waterfowl sharing environments where endemic leptospirosis occurred in other species of wildlife and domestic animals demonstrated that they were unimportant in the epidemiology of leptospirosis in the ecosystems under study. It was also shown that predator chain transmission of leptospires of the Ballum serogroup did not occur, despite the ecological dependance of mustelids and feral cats on endemically-infected rodent populations.

The widespread occurrence of Ballum serogroup infection in small free-living mammals in New Zealand, and the failure in the present study to isolate leptospires other than *balcanica* from other serogroups, is a unique consequence of the colonisation of vacant ecological niches by introduced species. Although Icterohaemorrhagiae serogroup infection is known to occur in Norway rats, the distribution of this serovar

appears to be restricted to localised foci. Endemic Ballum serogroup
infection in several free-living species sharing the same ecosystem
has not been reported in other countries. It is suggested that the
inadvertent introduction of serovars not already present in New Zealand
that are capable of being maintained by ship rats, hedgehogs and Norway
rats, could alter this situation. However, it is unlikely that endemic
Ballum serogroup infection in house mice, the internationally recognised
maintenance hosts for *ballum*, would be affected.

Endemic Ballum serogroup infection in free-living species will
continue to result in sporadic infections with these organisms in
domestic animals and humans in New Zealand. Unlike transmission
within a maintenance host population, infection is likely to be
transmitted indirectly via the environment or contaminated foodstuffs.
The limitations of environmental transmission of leptospirosis are
probably the major factors ensuring the Ballum serogroup infection in
domestic stock and man is a rare occurrence.

The ability of high population densities of Norway rats to maintain
an endemic focus of Ballum infection has public health implications that
should not be ignored. These rodents are generally restricted to
syanthropic foci. Changes in the environment supporting a high
population density of rats would result in the outward migration of
large numbers of infected animals and therefore increase the potential
for transmission of Ballum serogroup infection to humans. Control
programmes which are only imposed when populations reach high density
should therefore be avoided.

Field surveys and an integrated epidemiological investigation of
leptospirosis in an intensive farming environment provided detailed
evidence of the natural nidality of those serovars known to be present
in New Zealand. The serovars of important public health significance
in this country, *hardjo* and *pomona*, are maintained independantly by
domestic animals. Thus wildlife in New Zealand does not constitute a
similar public health problem to that occurring in many other countries.

The final investigation described in this thesis was that of the
concept of a maintenance host, using a laboratory mouse model. As a

consequence of these experimental studies, a maintenance host for a particular serovar was defined as an animal which is capable of acting as a natural source of infection for its own species. The most important experimental procedure determining that an animal species is a maintenance host for a particular serovar is the demonstration of natural transmission between individuals. Thus a maintenance host population is defined as a population of a species of animal which acts as a continuous reservoir of a serovar in a specific ecosystem. This is characterised by natural transmission of infection between individuals of the same and successive generations in the population. It is believed that any attempts to control leptospirosis in domestic animals and man must be based on a full knowledge of the maintenance host populations in the ecosystems under consideration.

# REFERENCES

Abdulla, P.K., Karstad, L., Fisher, N.A. (1962): Cultural and serological evidence of leptospirosis in deer in Ontario. Canad. Vet. J., 3: 71-75

Addamiano, E., Babudieri, B., Bussivello, G., Giuati, G., Salvi, A. (1960): Estr. Dend. Inst. Sup. Sanita., 22. Cited by Torten *et al* (1965).

Alexander, A.D., Beneson, A.S., Byrne, R.J., Rurico, S., Diaz-Rivera, S., Evans, L.B., Gochenour, W.S., Hall, H.E., Hightower, J.A., Jeffries, H., de Jesus, J., Martinez, E., Paniagua, M., Pons, J.A., Ramos-Morales, F., Rodriguez-Molina, R., Swisher, K.Y., Woodward, T.E., Yager, R.H. (1963): Leptospirosis in Puerto Rico. Z.R. 2: 153-196.

Alexander, A.D., Smith, H.O., Hiatt, C.W., Gleiser, C.A. (1956). Presence of haemolysin in cultures of pathogenic leptospires. Proc. Exp. Biol., 91: 205-211.

Alexander, A.D., Wood, O., Yancey, F., Byrne, R.J., Yagar, R.H. (1971): Cross-neutralisation of leptospiral hemolysins from different serotypes. Inf. Imm., 4: 154-159.

Al Saadi, M., Post, G. (1976): Rodent leptospirosis in Colarado. J. Wildl. Dis., 12: 315-317.

Alston, J.M., Broom, J.C. (1958): 'Leptospirosis in Man and Animals'. E.and S.Livingstone Ltd., Edinburgh.

Altava, V., Barrera, M., Marin, C. (1956): Rev. Sanidad. Hig. Publ. Madrid. 30: Cited by Babudieri (1958).

Ananyin, V.V. (1954): Pirirodnaja ocagovost leptospirosov. Zh. Zoo. 33: 3331

Andrews, R.D., Ferris, D.H. (1966): Relationships between movement patterns of wild animals and the distribution of leptospirosis. J. Wildl. Dis., 30: 131-134.

Andrews, R.D., Ferris, D.H., Hanson, L.E., Reilly, J.R. (1964): Leptospirosis in a cattle-deer association. Z.R., 3: 79-92.

Andrews, R.D., Reilly, J.R., Ferris, D.H., Hanson, L.F. (1965): Leptospiral agglutinins in sera from Southern Illinois Herpeto-fauna. Bull. Wildl. Dis. Ass., 1: 55-59.

Anon (1951): N.Z. Dept. Ag., Annual Report, 1950-51. 28p

Anon (1965): C.D.C. Zoonoses Surveillance, No 7. Special Report: Leptospirosis. U.S.D.H.E.W., Atlanta, Georgia.

Anon (1966): C.D.C. Zoonoses Surveillance. Leptospiral serotype distribution lists. U.S.D.H.E.W., Atlanta, Georgia.

Anon (1967): Current problems in leptospirosis. Wld. Hlth. Org. Tech. Rep. Ser., 380.

Anon (1967b): N.Z. Dept. Hlth., Annual Report. 1966-67. p41.

Anon (1973): Agricultural Research in N.Z. M.A.F., Annual Report of Research Division. 1972-73. p158.

Anon (1974): Surveillance, 1974. M.A.F., Wellington, N.Z. No 1. 28p.

Anon (1974b): Surveillance, 1974. M.A.F., Wellington, N.Z. No 2. 28p.

Anon (1975): C.D.C. Zoonoses Surveillance. Leptospiral serotype distribution lists according to host and geographic area. July, 1966 - July, 1973. U.S.D.H.E.W., Atlanta, Georgia.

Anon (1976): Surveillance, 1976. M.A.F., Wellington, N.Z. No 5. 28p.

Anon (1977): Proc. Marsupial Conference, organised by Victoria University Zoological Society, Wellington (In press).

Anon (1977b): Surveillance, 1977. M.A.F., Wellington, N.Z. No 5. 28p.

Atkinson, I.A.E. (1973): Spread of the ship rat (*Rattus rattus rattus L.*) in New Zealand. J. Roy. Soc. N.Z., $\underline{3}$: 457-72.

Audy, J.R. (1958): The localisation of disease with special reference to the zoonoses. Roy. Soc. Trop. Med. Hyg., $\underline{52}$: 308-328.

Auran, N.E., Johnson, R.C., Ritzi, D.M. (1972): Isolation of the outer sheath of Leptospira and its immunogenic properties in hamsters. Inf. Imm., $\underline{5}$: 968-975.

Babudieri, B. (1958): Animal resevoirs of leptospires. Ann. N.Y. Acad. Sci., $\underline{70}$: 393-413.

Babudieri, B. (1972): Systematics of a leptospira strain isolated from a frog. Experientia, $\underline{28}$: 1252.

Babudieri, B., Bianchi, C., (1940): Untersuchungen uber ein epidemisches Vorkommen der Reisfelder-leptospirosis in de Provinz Pavia. Zeitschr. Immf., $\underline{98}$: 37-75.

Babudieri, B., Bussinello, E., Bajocchi, E., Salvi, A., Massa, L. (1955): Stusisulla leptospirosi della risaie della Valle Padana Richerche epidemiologiche e profilassi vaccinale. Riv. Infort. Mal. Prof., $\underline{42}$: 453-484.

Babudieri, B., Mateev (1961): R.C. lst. sup. Sanita, $\underline{24}$: 614-622 Cited by Manev and Siromashkova (1970).

Baker, M.F., Baker, H.J. (1970): Pathogenic Leptospira in Malaysian surface waters. Amer. J. Trop. Med. Hyg., $\underline{19}$: 485-491.

Bamford, J.M. (1970): Evaluating opossum poisoning operations by interference with non-toxic baits. Proc. N.Z. Ecol. Soc., 17: 118-125.

Barrett, J.T. (1978): 'Textbook of Immunology' 3rd Ed., C.V. Mosby Co., St. Louis.

Batcheler, C.L. (1971): Estimation of density from a sample joint point and nearest-neighbour distances. Ecol. 52: 703-709.

Batcheler, C.L. (1973): Estimation of population density changes. In F.R.I. Symposium 14, N.Z. Forest Service.

Batcheler, C.L., Darwin, J.H., Pracy, L.T. (1967): Estimation of opossum (*Trichosurus vulpecula*) populations and results of poison trials from trapping data. N.Z.J. Sci., 10: 97-114.

Bentley, E.W., Taylor, E.J. (1965): Growth of laboratory-reared ship rats (*Rattus rattus* L.). Ann. Appl. Biol. 55: 193-205.

Best, L.W. (1968): The ecology of *Rattus rattus rattus* L. in selected areas of the South Island, New Zealand. Unpub. M.Sc. Thesis, University of Canterbury, New Zealand.

Best, L.W. (1972): Breeding season and fertility of the roof rat, *Rattus rattus rattus*, in two forested areas of New Zealand. N.Z.J. Sci., 16: 161-170.

Beveridge, T. (1964): Dispersal and destruction of seed in central North Island podocarp forests. Proc. N.Z. Ecol. Soc., 11: 48-55.

Bhasin, J.L., Freeman, M.J., Morter, R.L (1971): Properties of a cold hemagglutinin associated with leptospiral hemolytic anaemia of sheep. Inf. Imm., 13: 398-404.

Birnbaum, S., Shenberg, E., Torten, M. (1972): The influence of maternal antibodies in the epidemiology of the leptospiral carrier state in mice. Amer. J. Epidemiol., 96: 313-317.

Birnbaum, S., Torten, M., Shenberg, E. (1974): Experimental evidence for a cyclic occurrence of leptospirosis in vector mice. Amer. J. Epidemiol., 99: 225-229.

Blackmore, D.K. (1964): A survey of disease in British wild foxes. Vet. Rec., 76: 527-533.

Blackmore, D.K., Marshall, R.B., Ingram, B.R. (1976): An epidemiological investigation of leptospirosis at an artificial breeding centre. N.Z. Vet. J., 24: 253-262.

Blakelock, J.H., Allen, R.E. (1956): A survey of rats trapped in the Wellington area for ectoparasites and organisms of the Salmonella, Pasteurella and Leptospiral groups. N.Z. Vet. J., 4: 154-156.

Blood, B.D., Szyfres, B., Moya, V. (1963): Natural Leptospira pomona infection in the Pampas Cavy. Pub. Hlth. Rep., 78: 537-542.

Bloom, F. (1961): Chronic interstitial nephritis in the dog. Vet. Scope, 5: 2-9.

Boersma, A. (1974): Opossums in the Hokitika River catchment. N.Z.J. Forestry Sci., 4: 64-75.

Bollinger, A. (1946): Some aspects of marsupial reproduction. J. Roy. Soc. N.S.W., 80: 2-13.

Bollinger, A., Carrodus, A.L. (1938): Spermatorrhea in *Trichosurus vulpecula* and other marsupials. Med. J. Aust., 25: 1118-1119.

Borg-Petersen, C. (1944): *Leptospira ballum* , a new serological leptospira type? Acta. Path. Microbiol. Scand., 21: 504.

Borg-Petersen, C. (1949): Experience of leptospirosis in Denmark. Proc. Roy. Soc. Med., 42: 714-718.

Borg-Petersen, C., Fagroeus, A. (1949): The influence of the antigen density and other factors on the serum titre in the agglutination-lysis test for leptospirosis. Seperatum. Acta. Path., XXVI: 555-567.

Borg-Petersen, C., Fennestad, K.L. (1956): A field rodent (*Apodemus agrarius*) as a carrier of *Leptospira pomona* in Denmark. J. Amer. Vet. Med. Ass., 140: 204-205.

Borg-Petersen, C., Fennestad, K.L. (1962): Leptospiral infection in hedgehogs in Denmark. Acta. Path. Microbiol. Scand. Suppl., 154: 306.

Borg-Petersen, C., Fennestad, K.L. (1966): Leptospirosis in bats. Proc. Int. Congr. Parasitol., 1:404-405.

Braun, J.L., McCulloch, W.F., Top, F.H. (1964): Further observations on the epidemiology of leptospirosis in Iowa. Z.R. 3: 39-45.

Brockie, R.E. (1975): Isolation of *Leptospira hardjo* from the opossum (*Trichosurus vulpecula*). N.Z. Vet. J., 23: 216.

Brockie, R.E. (1975b): Distribution and abundance of the hedgehog (*Erinaceus europaeus*) in New Zealand. N.Z.J. Zoo. 2: 445-462.

Brockie, R.E. (1976): The role of wild animals in maintaining and transmitting leptospirosis. Unpub. Report, National Health Institute, Dept. Health, Wellington

Brockie, R.E. (1977): Leptospiral infections of rodents in the North Island. N.Z. Vet. J., 25: 89-91, 95.

Brockie, R.E., Till, D.G. (1977): *Leptospira ballum* isolated from hedgehogs. N.Z. Vet. J., 25: 28-30.

Broom, J.C. (1958): Leptospiral infection rates of wild rats in Britain. J. Hyg. (Lond), 56: 371-376.

Broom, J.C., Cohlan, J.D. (1958): *Leptospira ballum* in small rodents in Scotland. Lancet. Nov. 15th., 1041-1042.

Brown, R.Z., Gorman, G.W. (1960): The occurrence of leptospiral infections in feral rodents in Southwestern Georgia. Amer. J. Pub. Hlth., 50: 682-688.

Buddle, J.R., Hodges, R.T. (1977): Observations on some aspects of the epidemiology of leptospirosis in a herd of pigs. N.Z. Vet. J., 25: 65-66.

Burgdorfer, N. (1956): The possible role of ticks as vectors of leptospirosis. Exp. Parasit., 5: 571.

Butler, J.E. (1969): Bovine immunoglobulins: A review. J. Dairy Sci., 52: 1895-1909.

Calhoun, J.B. (1962): The ecology and sociology of the Norway rat. U.S.D.H.E.W., Pub. Hlth. Service Pub. No. 1008.

Calhoun, J.G. Casby, J.U. (1958): Calculation of the home range and density of small mammals. U.S. Pub. Hlth. Service. Pub. Hlth. Monograph 55. 24 pp.

Campbell, P.A. (1973): The feeding behaviour of the hedgehog *(Erinaceus europaeus* L.) in pasture land in New Zealand. Proc. N.Z. Ecol. Soc., 20: 35-40.

Campbell, R.S.F., Stallman, N.D. (1975): Bovine nephritis associated with *Leptospira autralis*. Aust. Vet. J., 51: 328.

Catchpole, M. (1934): Leptospiral jaundice in silver foxes. Vet. Rec., 14: 376-378.

Caughley, G. (1967): Calculation of population mortality rate and life expectancy for thar and kangaroos from the ratio of juveniles to adults. N.Z.J. Sci., 10: 578-584.

Chang, S.L. (1947): Studies of *Leptospira icterohaemorrhagiae*. I. Two new medium for growing *L. icterohaemorrhagiae*, *L. canicola* and *L. biflexa*, and a method for maintaining the virulence of *L. icterohaemorrhagiae* in culture. J. Infect. Dis., 81: 28-34.

Chang, A., Faine, S. (1974): Relative specificities of immunoglobulins reacting with axial filament antigens of Leptospira. Aust. J. Exp. Biol. Med., 52: 639-646.

Chernukcha, Y.G., Ananyina, Y.U., Zenkovitch, N.S. (1974): Pathogenicity of leptospires of various serological types for some species of wild rodents. Zbl. Bakt. Hyg., I. Abt. Orig. A., 228: 388-395.

Chernukha, Y.G., Kokovin, I.L., Ananyina, Y.V., Ryabov, N.I., Devyatova, A.P. (1975): Dependence of pathogenic properties of leptospires on animal subspecies. Zbl. Bakt. Hyg., I. Abt. Orig. A., 231: 175-179.

Chernukha, Y.G., Shiskina, Z.S. Baryshev, P.M., Kokovin, I.L. (1976). The dynamics of lgM- and lgG- antibodies in leptospiral infection in man. Zbl. Bakt. Hyg., I.Abt. Orig. A., 236: 336-343.

Chorvath, B. (1975): Some properties of leptospiral haemolysin. Biologia (Brat.), 30: 460-465.

Christmas, B.W., Tennent, R.B., Philip, N.A., Lindsay, P.G. (1974): Dairy farm fever in New Zealand: A local outbreak of leptospirosis. N.Z. Med. J., 79: 901-904.

Chung, G.T. (1968): Leptospiral infection of apparently normal pigs. Aust. Vet. J., 44: 315-319.

Chung Huei-Lan, Ch'iu fu Hsi, Wu Hsuen Tsung, Yeh Huei-fen, Hou Tsung-Ch'ang, Chang Nai-Cheng, Hsien Wei-Hsun, T'sao Wei-Chi. (1960): Leptospirosis: Epidemiological studies in the Kwantung province China. J. Hyg. Epidemiol. (Praha), 4: 347-362. Cited in Anon (1966).

Clark, I.G. (1961): Leptospirosis in Pennsylvania. A progress report. Proc. U.S. Livestock San. Ass.,65:140-146.

Clark, I.G., Varela-Diaz, V.M., Sulzer, C.R., Marshak, R.R., Hollister, C.J. (1966): Leptospirosis in Nicaragua: Preliminary report on the first year of study. Amer. J. Trop. Med. Hyg., 15: 735-742.

Cole, J.R., Sulzer, C.R., Pursell, A.R. (1973): Improved microtechnique for the leptospiral M.A.T., Appl. Microbiol. 25: 976-980.

Collier, W.A. (1948): Die ver breitung der Leptospiren in Neider-landisch-Indien. Acta Tropica, 5: 135. Cited in Anon., (1966).

Collier, W.A., Mochtar, A. (1939): Een serologisch afwijkende leptospira-stam uit de nier eener vleemis. Gen. Tijdschr. v. Ned. Ind., 79: 226. Cited in Anon., (1966).

Combiesco, D., Sturdza, N., Radu, I., Sefer, M. Nicolesco, I. (1959): Recherches concernant les sources d'infection in dans les leptospiroses. Arch. Roumaines Path.Exper. et Microbiol., 18: 361-371.

Corbould, A. (1972): Leptospirosis in Tasmania. Tas. J. Agri ., 43: 200-205.

Crawford, R.P. (1972): Identification of the immunoglobulins in serums of guinea pigs infected with serogroup Pomona leptospires. Amer. J. Vet. Res., 33: 2289-2298.

Crawley, M.C. (1970): Longevity of Australian brush-tailed opossums *(Trichosurus vulpecula)* in indigenous forest in New Zealand. N.Z.J. Sci., 13: 348-351.

Crawley, M.C. (1973): A live trapping study of Australian brush-tailed opossums, *Trichosurus vlupecula* (Kerr), in the Orongorongo Valley, Wellington, New Zealand. Aust. J. Zoo., 21: 75-90.

Daniel, M.J. (1966): A preliminary survey of the incidence of brucellosis, leptospirosis and salmonellosis in red deer in New Zealand. N.Z.J. Sci., 9: 399-408.

Daniel, M.J. (1967): A survey of diseases in fallow, Virginia and Japanese deer, chamois, tahr, and feral goats and pigs in New Zealand. N.Z.J. Sci., 10: 949-963.

Daniel, M.J. (1969): A survey of rats on Kapiti Island, New Zealand. N.Z.J. Sci., 12: 363-372.

Daniel, M.J. (1972): Bionomics of the ship rat *(Rattus r. rattus)* in a New Zealand indigenous forest. N.Z.J. Sci., 15: 313-341.

Daniel, M.J. (1973): Seasonal diet of the ship rat *(Rattus r. rattus)* in lowland forest in New Zealand. Proc. N.Z. Ecol. Soc., 20: 21-30.

Das Gupta, B.M. (1940): Natural leptospiral infection in the rat population of Calcutta. Ind. Med. Gaz., 75: 284-286.

Davidson, M.M. (1965): Changes in wild mammal populations in Canterbury. N.Z. Forest Service, Info. Series No. 52. 51pp.

Davis, D.E. (1964): Manual for analysis of rodent populations Pennsylvania State University Publication. 82pp.

Davis, D.E., Emlen, J.T., Stokes, A.W. (1948): Studies on home range in the brown rat. J. Mammal., 29: 207-225.

de Jong, H., Fowler, G.F. (1968): Unpublished. Cited in Anon (1975).

de Lisle, G.W., Almand, K.B., Julian, A.F., Wallace, J. (1975): Leptospirosis in the opossum *(Trichosurus vulpecula)*. N.Z. Vet. J. 23: 215-216.

Dice, L.R. (1938): Some census methods for mammals. J. Wildl.Mgmt. 2: 119-130.

Diesch, S.L., McCullough, W.F., Braun, J.L. (1966): Leptospires isolated from frog kidneys. Nature, 209: 939-946.

Dodd, D.C., Brakenridge, D.T. (1960): *Leptospira icterohaemorrhagiae* AB infection in calves. N.Z. Vet. J., 8: 71-76.

Doherty, P.C. (1966): Comparison of direct microscopic and guinea pig inoculation techniques for demonstrating leptospires in bovine urine. Aust. Vet. J., 42: 466-467.

Doherty, P.C. (1967): Bovine Leptospira pomona infection: Environmental contamination and the spread of the disease in a susceptible herd. Queens. J. Ag. An. Sci., 24: 329-341.

Duncan J.R., Wilkie, B.N., Hiestand, F., Winter, A.J. (1972): The serum and secretory immunoglobulins of cattle: Characterisation and quantitation. J. Immunol., 108: 965-976.

Dunnett, G.M. (1956): A live-trapping study of the brush-tailed possum *Trichosurus vulpecula* Kerr (Marsupialia).C.S.I.R.O. Wildlife Res., 1: 1-16.

Durfee, P.T., Presidente, P.J.A. (1977): Isolation of *Leptospira interrogans* serotype *balcanica* from a brush-tailed possum *(Trichosurus vulpecula)*. Aust. Vet. J., 53: 508.

Ellinghausen, H.C. (1976): Nutrition of leptospires in bovine albumin polysorbate medium. In 'Biology of Parasitic Spirochetes'. Academic Press (Inc.), New York.

Ellinghausen, H.C., McCullough, W.G. (1965): Nutrition of *Leptospira pomona* and growth of 13 other serotypes: Fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Amer. J. Vet. Res., 26: 45-51.

Ellinghausen, H.C., Painter, G.M. (1976): Growth, Survival, antigenic stability and virulence of *Leptospira interrogans* serotype *canicola*. J. Med. Microbiol., 9: 29-37.

Ellinghausen, H.C., Sandvik, O. (1965): Tributyrinase activity of leptospires: fixed and soluble tributyrinase demonstrated by means of an agar diffusion test. Acta. Path. Microbiol. Scand., 65: 259-270.

Ellis, W.A., Mich a, S.W. (!976): Bovine leptospirosis: Demonstration of leptospires of the Hebdomadis serogroup in aborted foetuses and a premature calf. Vet. Rec., 99: 430-432.

Ellis, W.A., Michna, S. W. (1977): Bovine leptospirosis; Experimental infection of pregnant heifers with a strain belonging to the Hebdomadis serogroup. Res. Vet. Sci., 22: 229-236.

Emanuel, M.L. (1959): The susceptibility of mice to North Queensland strains of leptospirae. Aust. J. Exp. Biol., 37: 17-30.

Emanuel, M.L., Mackerras, I.M., Smith, D.J.W. (1964): The epidemiology of leptospirosis in North Queensland. (1) General survey of animal hosts. J. Hyg., Camb., 62: 451-484.

Esseveld, H. (1948): Leptospirosis in Java and Sumatra. J. Trop. Med. Hyg., 51: 171. Cited in Anon (1966).

Faine, S. (1960): Catalase activity in pathogenic Leptospira. J. Gen. Microbiol., 22: 1-9.

Faine, S. (1962): The growth of *Leptospira australis* B in the kidneys of mice in the incipient experimental carrier state. J. Hyg. Camb., 60: 435-442.

Faine, S., Kirschner, L. (1953): Human leptospirosis in New Zealand, 1951-52. N.Z. Med. J., 52: 12-14.

Fennestad, K.L. (1963): 'Experimental Leptospirosis in calves'. Munksgaard, Copenhagen.

Fennestad, K.L., Borg-Petersen, C. (1972): Leptospirosis in Danish wild mammals. J. Wildl. Dis., 8: 343-351.

Ferris, D.H., Andrews, R.D. (1967): Parameters of a natural focus of *Lepsospira pomona* in skunks and opossums. Bull. Wildl. Dis. Ass., 3: 2-10.

Ferris, D.H., Rhoades, H.E., Hanson, L.E., Galton, M., Mansfield, M.E. (1961): Research into the nidality of *Leptospira ballum* in campestral hosts including the hog-nosed snake *(Heterodon platyrhinus)*. Cornell Vet., L1: 405-419.

Ferris, D.H. Verts, B.J. (1964): Leptospiral reactor rates among white-tailed deer and livestock in Carroll County, Illinois. J. Wildl. Mgmt., 28: 35-41.

Fitzgerald, B.M., Karl, B.J. (1977): Rodent populations in the Orongorongo Valley, Wellington, 1971-1976. Proc. N.Z. Ecol. Soc., 24: 127-128.

Fletcher, W. (1928): Recent work on leptospirosis, tsutsugamushi disease and tropical typhus in the Federated Malay States. Trans. Roy. Soc. Trop. Med. Hyg., 21: 265-287. Cited by Sulzer and Jones (1974).

Fraga de Azevedo, J., Valente, J.S., Queiros, J.J. deS. (1951): Novos dados sobre as Leptospiras de Portugal. Anais. Inst. Med. Trop., 8: 621. Cited by Borg-Petersen and Fennestad (1956).

Fowler, G. (1970): Axenic cultures of leptospires. N.Z. Vet. J., 18: 202.

Gale, W.B. (1965): Pers. comm., cited in Anon (1966).

Galton, M.M., Menges, R.W., Shotts, E.B., Nahamias, A.J. Heath, C.W. (1962): Leptospirosis: Epidemiology, clinical manifestations in man and animals, and methods in laboratory diagnosis. U.S. Public Health Service Pub. No. 951, Washington D.C.

Galton, M.M., Sulzer, C.R., Santa Rosa, C.A., Fields, M.J. (1965): Application of a microtechnique to the agglutination test for leptospirosis antibodies. Appl. Microbiol., 13: 81-85.

Gillespie, R.W.H., Kenzy, S.G, Ringen, L.M., Bracker, F.K. (1957): Studies on bovine leptospirosis. III. Isolation of *Leptospira pomona* from surface waters. Amer. J. Vet. Res., 18: 76-80.

Gillespie, R.W.H., Ryno, J. (1963): Epidemiology of leptospirosis. Amer. J. Pub. Hlth., 53: 950-955.

Gilmore, D.P. (1965): Opossums eat pasture. N.Z.J. Ag., 110: 284-286.

Gilmore, D.P. (1965b): Food of the possum *Trichosurus vulpecula* in pastoral areas of Banks Pennisula, Canterbury. Proc. N.Z. Ecol. Soc., 12: 10-13.

Gilmore, D.P. (1969): Seasonal reproductive periodicity in the male Australian brush-tailed possum *(Trichosurus vulpecula)*. J. Zoo., Lond., 157: 75-98.

Glosser, J.W., Sulzer, C.R., Eberhardt, M., Winkler, W.G. (1974): Cultural and serological evidence of *Leptospira interrogans* serotype *tarassovi* infection in turtles. J. Wildl. Dis., 10: 429-435.

Gordon, L.M. (1977): *Leptospira interrogans* serotype *hardjo* outbreak in a Victoria dairy herd and associated infection in man. Aust. Vet. J., 53: 227-229.

Gordon, Smith, C.E., Turner, L.H. (1961): The effect of pH on the survival of leptospires in water. Bull. Wld. Hlth. Org., 24: 35-43.

Gordon-Smith, C.E., Turner, L.H. Harrison, J.L. Broom, J.C. (1961): Animal leptospirosis in Malaya. I. Methods, zoogeographical background and broad analysis of results. Bull. Wld. Hlth. Org ., 24: 5-21.

Gordon-Smith, C.E., Turner, L.H. Harrison, J.L. Broom, J.C. (1961b): Animal leptospirosis in Malaya. 2: Localities Sampled. Bull. Wld. Hlth. Org., 24: 23-24.

Gordon-Smith, C.E. Turner, L.H., Harrison, J.L., Broom, J.C. (1961c): Animal leptospirosis in Malaya. 3: Incidence in rats by sex, weight and age. Bull. Wld. Hlth. Org., 24: 807-816.

Grant, P.J. (1956): Opossum damage in the beech forests, Ruahine Range, Hawkes Bay. N.Z.J. Forestry, 7: 111-112.

Graves, S., Faine, S. (1970): Antileptospiral agglutinins produced in rabbits. Bull Wld. Hlth. Org., 43: 579-587.

Gsell, O. (1952): 'Lepsospirosen'. Huber, Bern. Cited in Anon (1966).

Hamdy, A.H., Ferguson, L.C. (1957): Virulence of *Leptospira pomona* in hamsters and cattle. Amer. J. Vet. Res., 18: 45-51.

Hanson, L.E. (1973): Immunologic problems in bovine leptospirosis. J. Amer. Vet. Med. Assoc., 163: 919-921.

Hanson, L.E. (1976): Bovine leptospirosis. J. Dairy Sci., 59: 1166-1170.

Hanson, L.E. (1977): Immunology of bacterial diseases, with special reference to leptospirosis. J. Amer. Vet. Med. Ass., 170: 991-994.

Hanson, L.E., Ellinghausen, H.C., Marlow, P. (1964): Isolation of
    *Leptospira grippotyphosa* from a cow following an abortion.
    Proc. Soc. Biol. Med. 17: 495-497.

Hanson, L.E., Reynolds, H.A., Evans, L.B. (1971): Leptospirosis in
    swine caused by serotype *grippotyphosa*. Amer. J. Vet. Res.,
    32: 855-860.

Hanson, L.E., Tripathy, D.N., Killinger, A.H. (1972); Current
    status of leptospirosis immunisation in swine and cattle.
    J. Amer. Vet. Med. Ass., 161: 1235-1242.

Harkness, A.C., Smith, B.L., Fowler, G.F. (1970): An isolation
    of *Leptospira* serotype *pomona* from a domestic cat. N.Z. Vet. J.
    18: 175-176.

Hartley, W.J. (1952): Ovine leptospirosis. Aust. Vet. J., 28: 169-170.

Hartmann, L., Filitti-Wurmser, S., Jacquot-Armand, Y., Mailloux, M.,
    Hurez, D., Fauvert, R. (1964): Nature macromoleculaire d'un
    anticorps de la leptospirose *australis*. Biochem. Biophys.
    Acta, 82: 249-259.

Harvey, A.E. (1973): Diet of the opossum (*Trichosurus vulpecula*
    Kerr) on farmland north-east of Waverley, New Zealand.
    Proc. N.Z. Ecol. Soc., 20: 48-52.

Hellstrom, J.E. (1978): Studies on some aspects of the epidemiology
    of bovine leptospirosis. Unpub. Ph.D. Thesis, Massey University
    Palmerston North.

Hirschberg, N., Vaughn, J. (1973): Antibodies in bovine urine in
    leptospirosis. Vet. Med/S.A.C., 68: 69-74.

Hoare, R.J., Claxton, P.D. (1972): Observations on *Leptospira
    hardjo* infection in New South Wales. Aust. Vet. J., 48:
    228-232.

Hocker, N.D., Bauer, C.D. (1965): The nature of antibodies synthesised
    during the immune responses to *Leptospira biflexa*. J. Immunol.,
    95: 887-894.

Hodges, R.T. (1974): Bovine leptospirosis: the detection of
    haemolytic inhibitors in sera from experimentally and
    naturally infected cattle. N.Z. Vet. J., 22: 239-242.

Hodges, R.T. (1977): *Leptospira interrogans* serotype *pomona* infection
    in pigs: prevention of leptospiruria by immunisation before
    exposure to natural infection. N.Z. Vet. J. 25: 33-35.

Hodges, R.T., Ekdahl, M.O. (1973): Use of a fluorescent antibody
    technique for the serological differentiation of leptospiral
    serotypes in cultures and in bovine urine. N.Z. Vet. J.,
    21: 109-115.

Holloway, J.T. (1959): Noxious animal problems of the South Island
    alpine watersheds. N.Z. Sci. Rev., 17: 2-10.

Howard, W.E., (1964): Modification of New Zealands flora by
    introduced mammals. Proc. N.Z. Ecol. Soc., 11: 59-62.

Howell, J.C. (1954): Populations and home ranges of small mammals
    in an overgrown field. J. Mammal., 35: 177-186.

Hussaini, S.N., Ruby, K.R. (1976): Comparative studies on the
    use of 5-fluorouracil in two different media as a selective
    agent for isolation of leptospira. Res. Vet. Sci., 20: 148-150.

Ido, Y., Hoki, R., Ito, H., Wani, H. (1917): The rat as a carrier of
    Spirochaeta icterohaemorrhagiae, the causative agent of
    Weil's disease (Spirochaetosis ictero haemorrhagica).
    J. Exp. Med. 26: 341-353.

Inada, R., Ido, Y., Hoki, R. Kaneko, R., Ito, H. (1916): The
    etiology, mode of infection and specific therapy of Weil's
    disease (Spirochaetosis icterohaemorrhagica). J. Exp. Med.
    23: 377-402.

Innes, J. (1977): Biology and ecology of the ship rat, *Rattus rattus
    rattus* (Lynn) in Manawatu (N.Z.) forests. Unpub. M.Sc. Thesis,
    Massey University, Palmerston North.

Jamieson, S., Davidson, R.M., Salisbury, R.M.. (1970): Leptospirosis
    in New Zealand. Bull. Int. Epiz., 73: 81-92.

Johnson, R.C., Gary, N.D. (1962): Nutrition of *Leptospira pomona*.
    I. Studies on a chemically-defined substitute for the rabbit
    serum ultrafiltrate. J. Bact., 83: 668-672.

Johnson, R.C., Gary, N.D. (1963a): Nutrition of *Leptospira pomona*.
    II. Fatty acid requirements. J. Bact., 85: 976-982.

Johnson, R.C., Gary, N.D. (1963b): Nutrition of *Leptospira pomona*
    III. Calcium, magnesium and potassium requirements.
    J. Bact., 85: 983-985.

Johnson, R.C., Harris, V.G. (1967): Differentiation of pathogenic
    and parasitic leptospires. I. Growth at low temperatures.
    J. Bact., 94: 27-31.

Johnson, R.C., Rogers, P. (1964): 5-Fluorouracil as a selective
    agent for growth of leptospirae. J. Bact., 88: 422-427

Johnson, R.H., Allan, P.J., Dennett, D.P. (1974): Association of
    *Leptospira hardjo* with abortions in a group of heifers.
    Aust. Vet. J., 50: 325-326.

Jolly, J.N. (1973): Movements and social behaviour of the opossum,
    *Trichosurus vulpecula*, Kerr, in a mixed scrub, bush and
    pasture habitat. Mauri Ora, 1: 65-71.

Jolly, J.N. (1976): Habitat use and movements of the opossum
(*Trichosurus vulpecula* ) in a pastoral habitat on Banks Peninsula.
Proc. N.Z. Ecol. Soc., 23: 70-78.

Josland, S.J., Allen, R.E., Cashmore, S., Scott, H.M. (1957): Survey
work on human leptospirosis in New Zealand. N.Z. Med. J., 56:
128-131.

Kallai, L., Kemenes, F., Vizy, L. (1962): Studies on the *Leptospira
icterohaemorrhagiae* infection of experimental rats. Acta.
Microbiol. Hung., 9: 311-315.

Karaseva, E.V., Chernukha, Y.G., Piskunova, L.A. (1973): The results
of studying the time of survival of pathogenic leptospira
under natural conditions. J. Hyg. Epidemiol. Microbiol.,
17: 339-345.

Kasarov, L.B. (1976): Degradation of the erythrocyte phospholipids
and haemolysis of the erythrocytes of different animal species
by leptospirae. J. Med. Microbiol., 3: 807-815.

Kathe, J. (1950): Zbl. Bakt. Parasit. Orig. 155: 199. Cited by
Babudieri (1958).

Kaufmann, A.F. (1976): Epidemiological trends of leptospirosis in
the United States. In 'Biology of the parasitic Spirochetes'.
Academic Press (Inc.), New York.

Kean, R.I. (1959): Bionomics of the brush-tailed opossum (*Trichosurus
vulpecula*) in New Zealand. Nature, 184: 1388-1389.

Kean, R.I. (1967): Behaviour and territorialism in *Trichosurus
vulpecula* (Marsupialia). Proc. N.Z. Ecol. Soc., 14: 71-78.

Kean, R.I. (1971): Selection for melanism and for low reproductive
rate in *Trichosurus vulpecula* (Marsupialia). Proc. N.Z. Ecol.
Soc., 18: 42-47.

Keast, J.C., Littlejohns, I.R., Rowan, L.C., Wannan, J.S. (1966):
The role of the feral pig as a disease resevoir. Aust. Vet.
J., 39: 99.

Kemenes, F. (1957): Untersuchungen der Leptospirenin fektionen von
nagetieren in einer gemeide von sudostungarn. Acta. Microbiol.
Acad. Sci. Hung., 4: 167. Cited in Anon (1966).

Kemenes, F. (1964): Cross-immunity studies or virulent strains
of leptospires belonging to different serotypes. Zeitschr. f.
Immunitats-u-Allergieforsch, 127: 209-229.

Kemenes, F. (1974): Biological characteristics of hemolysin from
*Leptospira pomona*. Folia Fac. Med. Univ. Comenianae
Bratisl., 12: Supplementum, 1974.

Kemenes, F., Szeky, A. (1966): *Leptospira sejro* infection of albino mice in Hungary. (Eradication of leptospires from infected mouse stocks.) Zbl. Vet., 13: 591-600.

Kenzy, S.G., Gillespie, R.W.H. (1957): Failure of infected ducks to transmit *Leptospira pomona* to calves. Vet. Med. 52: 310-311.

Kiktenko, V.S., Balashov, N.G., Rodina, V.N. (1976): Leptospirosis infection through insemination of animals. J. Hyg. Epidemiol., Microbiol. Immunobiol., 20: 207-213.

Killinger, A.H., Taylor, P.C., Huhn, R.G., Hanson, L.E., Mansfield, M.E. (1976): Immunity to leptospirosis: Renal changes in vaccinated cattle given challenge inoculum. Amer. J. Vet. Res. 37: 93-94.

Kingscote, B.F. (1970): Correlation of bedrock type with geography of leptospirosis. Canad. J. Comp. Med., 34: 31-37.

Kirscher, L., Gray, W.G. (1951): Leptospirosis in New Zealand. Infection with spirochaetes in animals and man. N.Z. Med. J., 50: 342-351.

Kirschner, L., Maguire, T. (1957): Survival of leptospirae outside their hosts. N.Z. Med. J., 56: 385-391.

Kirschner, L., Miller, T.F., Garlick, C.H. (1952): Swineherds disease in New Zealand. Infection with *Leptospira pomona* in man, calves and pigs. N.Z. Med. J., 51: 98-108.

Kitoaka, M. (1951): Classification of leptospira. Jap. Med. J., 4: 181-214.

Kmety, E. (1955): Leptospirosenherde in der Slowakei. Zbl. Bakt. I. Orig., 163: 464-468.

Kmety, E. (1974): Problems of the serological classification of pathogenic leptospires. Folia Fac. Med., Univ. Comenianae Bratisl., 12: Supplementum, 1974.

Kmety, E., Bakoss, P. (1961): Hamolysin-und lipaseproduktion bei leptospirosen verschiedener serotypes. Zbl. Bakt. I. Abs. Orig. 181: 514-515.

Kmety, E., Galton, M.M., Sulzer, C.R. (1970): Further standardisation of the agglutinin-absorption test in the serology of leptospires. Bull. Wld. Hlth. Org., 42: 733-738.

Kmety, E., Plesko, I., Chylo, E. (1956): Weitere ergebnisse der Leptospirosenforschung in der Slowakei. Zbl. Bakt. I. Orig., 167: 243-253.

Korenberg, E.L., Pchelkina, A.A., Soloshenko, I.Z., Dunaeva, T.N. (1975): A study of red deer (*Cervus elaphus*) in combined foci of tick-borne encephalitis, rickettsial diseases, leptospiroses and tularaemia. Zh. Zoo., LIV: 1057-1066.

Krepkogorskaia, T.A., Rementsora, M.M. (1957): The isolation of strains of leptospires from the tick *Dermacentor marginatus* from cattle. J. Microbiol. Epidemiol. Immunobiol., 28: 25. Abstract in Vet. Med., 52: 545 (1957).

Kunert, H., Schmidtke, L. (1952): Diebedeutung der nichstechenden fliegen fur die Verschl-leppung von leptospiren. Z. Tropenmed. U. Parasit., 3: 475. Cited by Alston and Broom (1958).

Lahiri, M.N. (1941): Studies on *icterohaemorrhagiae* in rats in Bombay city. Ind. Med. Gaz., 76: 536-538.

Lake, D.E. (1973): Bovine leptospirosis. N.Z. Vet. J., 21: 52.

Lane-Petter, W. (1976): The laboratory mouse. In 'The UFAW Handbook on the care and management of laboratory animals'. 5th Ed. Churchill Livingstone, Edinburgh.

Langham, R.F., Morse, E.V., Morter, R.L. (1958): Pathology of experimental bovine leptospirosis, *Leptospira pomona* infection. J. Infect. Dis., 103: 285-290.

Little, T.W.A., Salt, G.F.H. (1975): Isolation of a leptospira of the 'Pomona' serogroup from a field vole. Vet. Rec., 97: 56.

Liven, E. (1975): Deoxyribonuclease production by leptospires. Acta Vet. Scand., 16: 477-479.

Locke, R.F., Segre, D., Myers, W.L. (1964): The immunologic behaviour of baby pigs. IV. Intestinal absorption and persistence of 6.6S 18S antibodies of ovine origin and their role in the immunologic competence of baby pigs. J. Immunol., 93: 576-581.

Lyne, A.G., Pilton, P.E., Sharman, G.B. (1959): Oestrus cycle, gestation period and parturition in the marsupial *Trichosurus vulpecula*. Nature, 183: 622-623.

Lyne, A.G., Verhagen, A.M.W. (1957): Growth of the *Trichosurus vulpecula* and a comparison with some higher mammals. Growth, 21: 167-195.

McCaughey, W.J., Fairley, J.S. (1971): Leptospirosis in Irish wildlife. Vet. Rec., 86: 447.

McDiarmid, A. (1965): Modern trends in animal health and husbandry. Some infectious diseases of free-living wildlife. Br. Vet. J. 121: 245-257.

McDiarmid, A. (1975): Some disorders of wild deer in the United Kingdom. Vet. Rec., 97: 6-9.

McGowan, J.E., Karstad, L. (1965): Field and laboratory studies of skunks, raccoons, and ground hogs as reservoirs of *Leptospira pomona*. Canad. Vet. J., 6: 243-252.

McGuire, T.C., Pfeiffer, N.E., Weikel, J.M., Bartsch, R.C. (1976):
Failure of colostral immunoglobulin transfer in calves
dying from infectious disease. J. Amer. Vet. Med. Ass.,
169: 713-718.

McIlhalton, T.J., Martin, J.W., Wagner, R.J., Iverson, J.O. (1971):
Isolation of *Leptospira pomona* from a naturally infected
California sea lion, Somona County, California. J. Wildl.
Dis., 7: 195-197.

Mailloux, M. (1975): Leptospiroses = Zoonoses. Int. J. Zoo., 2:
45-54.

Manev, C. (1976): Serological characteristics of the Leptospira
serogroup Pomona. II. Changes in the agglutination and
absorption properties of the reference strains after
formation and heat inactivation. Zbl. Bakt. Hyg. I. Abt.
Orig. A., 236: 323-327.

Manev, C., Siromashkova, M. (1970): Comparative studies on antigen
extract from some Bulgarian Leptospiral strains. Zbl. Bakt.
Parasit. Infekt. Hyg. I. Orig., 213: 526-532.

Manev, C., Yanakieva, M. (1973): Typing of Leptospira strains of
the Hebdomadis serogroup with absorped sera. Zbl. Bakt. Hyg. I.
Abt. Orig. A., 223: 228-234.

Marshall, R.B., Manktelow, B.W., Ryan, T.J., Hathaway, S.C. (1976):
*Leptospira interrogans* serovar *balcanica* from a possum.
N.Z. Med. J., 84: 74-75.

Marshall, W.H. (1963): The ecology of Mustelids in New Zealand.
N.Z. D.S.I.R. Infor. Series No. 38.

Martin, R.I., Hanson, L.E. Schnurrenberger, P.R. (1967): Leptospiral
interspecies infections on an Illinois farm. Pub. Hlth.
Rep., 82: 75-83.

Masaev, M.A. (1960): Environmental factors influencing the
incidence of bovine leptospirosis. Veterinariya, 37: 22-25.
Abstracted in Vet. Bull. (1960), 30: 491.

Mateev, D., Manev, C. (1974): On the etiological structure of the
leptospiroses in Bu lgaria. Folia. Fac. Med. Univ.
Comenianae Bratisl., 12: Supplementum, 1974.

Matveeva, A.A., Sakharova, P.U., Sharban, E.K. Dragomir, A.V. (1977):
Aetiology of leptospirosis in animals (cattle, swine and
small animals) in the Moldavian Republic, USSR. Veterinariya,
61: 63. Abstracted in Vet. Bull. (1977), 47: 590.

Meads, M.J. (1976): Effects of opossum browsing on northern rata in
the Orongorongo Valley, Wellington, New Zealand. N.Z.J. Zoo.,
3: 127-139.

Medinskii, G.M. (1959): Micro-focal occurrence of leptospirotic
        infection in the conditions of the Estonian SSR. J. Microbiol.,
        Epidemiol. Immunobiol., 30: 92-97.

Menges, R.W., Galton, M.M., Habermann, R.T. (1960): Culture and
        serologic studies on four dogs inoculated with two lepto-
        spiral serotypes, *Leptospira pomona* and *Leptospira canicola*.
        Amer. J. Vet. Res., 21: 371-376.

Michna, S.W. (1970): Leptospirosis. Vet. Rec., 86: 484-496.

Michna, S.W., Campbell, R.S.F. (1970): Leptospirosis in wild
        animals. J. Comp. Path., 80: 101-106.

Michna, S.W., Ellis, W. (1974): The isolation of Leptospira
        belonging to the serogroup *Ballum* from the kidneys of a
        brown rat (*Rattus norvegicus*). Res. Vet. Sci., 16: 263-
        264.

Minette, H.P. (1964): Leptospirosis in rodents and mongooses on
        the Island of Hawaii. Amer. J. Trop. Med. Hyg., 13: 826-832

Minette, H.P. (1966): Leptospirosis in primates other than man.
        Amer. J. Trop. Med. Hyg., 15: 190-198.

Minette, H.P., Shaffer, M.F. (1968): Experimental leptospirosis in
        monkeys. Amer. J. Trop. Med. Hyg., 17: 202-212.

Mino, P. (1939): Ueber Leptospirosen bei den arbeitern der
        reisfelder Oberitaliens. Zeitschr. Immf., 96: 466-485.
        Cited in Anon (1966).

Mitchell, D. Robertson, A., Corner, A.H. Boulanger, P. (1966):
        Some observations on the diagnosis and epidemiology of
        leptospirosis in swine. Canad. J. Comp. Med. Vet. Sci.,
        30: 211-217.

Mohn, S.V., Sandvik, O. (1976): Serological investigations of
        leptospiral deoxyribonucleases. Acta Vet Scand., 17: 354-358.

Morris, I.G. (1967): The transmission of bovine anti-*Brucella
        abortus* agglutinins across the gut of suckling rats.
        Immunol., 17: 139-146.

Morris, J.A., Hussaini, S.N. (1974): Characterisation of the
        antibodies detected by the microscopic agglutination test
        for bovine leptospirosis. J. Hyg. Camb., 73: 425-432.

Morrison, W.I., Wright, N.G. (1976): Canine leptospirosis: An
        immunopathological study of interstitial nephritis due to
        *Leptospira canicola*. J. Path., 120: 83-89.

Morse, E.V., Allen, V., Krohn, A.F., Hall, R. (1955): Leptospirosis
        in Wisconsin. I. Epizootiology and clinical features.
        J. Amer. Vet. Med. Ass., 127: 417-421.

Morse, E.V., Bauer, D.C., Langham, R.F., Lang, R.W., Ullrey, D.E., (1958): Experimental leptospirosis. IV. Pathogenesis of Porcine *Leptospira pomona* infections. Amer. J. Vet. Res., 19: 388-394.

Mosby, H.S. (1960): 'Manual of game investigational techniques'. Wildlife Society, Washington, D.C.

Munday, B.L. (1972): A serological study of some infectious diseases of Tasmanian Wildlife. J. Wildl. Dis., 8: 169-175.

Munday, B.L., Corbould, A. (1973): *Leptospira pomona* infection in wombats. J. Wildl. Dis., 9: 72-73.

Murnane, T.G., Alexander, A.D., Murphy, L.C., Evans, L.B., Gerardino, M.H. (1963): The occurrence of leptospiral antibodies in cattle in Panama. Z.R., 2: 83-90.

Murphy, L., Cardeilhac, P., Carr, J. (1958): The prevalence of leptospiral agglutinins in sera of domestic cats. Cornell. Vet., 48: 3-10.

Negi, S.K., Myers, W.L., Segre, D. (1971): Antibody response of cattle to *Leptospira pomona*: A hemagglutination test to measure IgG and IgM antibodies. Am. J. Vet. Res., 32: 1907-1913.

Nervig, R.M., Ellinghausen, H.C. (1978): Viability of *Leptospira interrogans* serotype *grippotyphosa* in swine urine and blood. Cornell. Vet., 68: 70-77.

Nityananda, K., Harvey, T. (1971): Leptospirosis in Ceylon. Epidemiological and laboratory studies. Ceylon J. Med. Sci., 20: 5-14.

Noguchi, H. (1917): Spirochaeta icterohemorrhagiae in American wild rats and its relation to the Japanese and European strains. J. Exp. Med., 25: 755-763.

Noguchi, H. (1918): Morphological characteristics and nonemclature of Leptospira (Spirochaeta) icterohaemorrhagiae (Inada and Ido). J. Exp. Med., 27: 575-592.

Okazaki, W., Ringen, L.M. (1957): Some effects of various environmental conditions on the survival of *Leptospira pomona*. Amer. J. Vet. Res., 18: 219-223.

Palit, A., Sharma, G.L. (1971): Comparison of microscopic agglutination, indirect haemagglutination and complement-fixation tests in rabbit and buffalo-calf hyperimmune sera for detection of leptospiral antibodies. Br. Vet. J., 127: 154-160.

Parkes, J.P. (1972): Some aspects of the biology of the hedgehog, *Erinaceus europaeus* L. in the Manawatu, New Zealand. Unpub. M.Sc. Thesis, Massey University, Palmerston North.

Parnas, J., Koslak, A., Cybulska, M. (1964): A characteristic of a natural leptospirosis focus found along the Polish-Russian confine and its liquidation. II. Int. Sym. 'Leptospirae and leptospiroses in men and animals'. Dec. 6-8, 1962. Lublin. P.Z.W.L., Warszawa, 1964. Cited in Anon (1966).

Parnas, J., Koslak, A., Krukowska, M. (1961): Leptospiren, die bei der hausmaus *(Mus musculus)* au ftreten. Zbl. Bakt., 182: 121-127.

Pearson, J. (1938): The Tasmanian brush opossum: its distribution and colour variations. Proc. Roy. Soc. Tasmania, (1937): 21-29.

Philip, N.A. (1976): Leptospirosis: New Zealands No. 1 dairy occupational disease. N.Z. Vet. J., 24: 6-8.

Pike, R.M. (1967): Antibody heterogenicity and serologic reactions. Bact. Rev., 31: 157-174.

Pike, R.M., McBrayer, H.L., Schulze, M.L., Chandler, C.H. (1965): Chromatographic analysis and sulfhydryl sensitivity of antileptospira agglutinins in rabbit and human sera. Proc. Soc. Exp. Biol. Med., 120: 786-789

Pilton, P.E., Sharman, G.B. (1962): Reproduction in the marsupial, *Trichosurus vulpecula*. J. Endocrin., 25: 119-136.

Pinto, A.A., Santa-Rosa, C.A., Sadatsure, T., Fleury, G.C. (1974): Comparative studies between complement fixation and microscopic agglutination tests for leptospiral diagnosis. Rev. Inst. Med. Trop., Sao Paulo, 16: 28-31.

Plesko, I. (1974): Cross-immunity relations among lipase positive and negative leptospiral strains. Biologia (Bratisl.), 29: 705-710.

Plesko, I., Novakova, Z. (1967): Nore poznatky 20 sledovania resevoarov leptospirna juhozapudnom Slovensky. Bratial. lek. Lisly, 48: 115. Cited in Anon (1966).

Popova, E.M., Amonsenkova, N.I. (1957): Hosts of field fever in the Northwest part of the USSR. Z. Mikrobiol., 1: 44. Cited in Anon (1966).

Popp, L. (1950): Z. Hyg. Infektionskrankh, 131: 575. Cited by Babudieri (1958).

Porter, P. (1972): Immunoglobulins in bovine mammary secretions. Quantitative changes in early lactation and absorption by the neonatal calf. Immunol., 23: 225-238.

Pracy, L.T. (1962): Introduction and liberation of the opossum *(Trichosurus vulpecula)* into New Zealand. N.Z. Forest Service, Info. Series No. 45. 28pp.

Pracy, L.T., Kean, R.I. (1969): The opossum in New Zealand. Habits and trapping. N.Z. Forest Services Publicity Item No. 40. 52pp.

Quinn, J. (1968): Opossums deplete Wanganui pastures. N.Z.J. Ag., 117: 17-20.

Reilly, J.R. (1970): The susceptibility of five species of wild animals to experimental infection with *Leptospira grippotyphosa*. J. Wildl. Dis., 6: 289-294.

Reilly, J.R., Muraschi, T.F., Dean, D.J. (1962): Leptospirosis in the white-tailed deer (*Odocoileus virginianus*). Cornell Vet., L11: 94-98.

Reilly, J.R., Hanson, L.E., Ferris, D.H. (1970): Experimentally-induced predator chain transmission of *Leptospira grippotyphosa* from rodents to wild Marsupialia and Carnivora. Amer. J. Vet. Res., 31: 1443-1448.

Rimpau,W. (1942): Systematische untersuchungen von feldmausen auf leptospiren. Munch. Med. Wschr., 2: 991. Cited in Anon (1966).

Ris, D.R., Lake, D.E., Holland, J.T.S. (1973): The isolation of *Leptospira* serotypes *copenhageni* and *ballum* from healthy calves. N.Z. Vet. J., 21: 218-220.

Roth, E.E. (1961): Leptospirosis in striped skunks. Unpub. M.Sc. Thesis, Agricultural and Mechanical College of Texas.

Roth, E.E. (1970): Leptospirosis. In 'Infectious diseases of wild mammals.' Iowa State University Press, Ames.

Roth, E.E., Adams, W.V., Sanford, G.E., Greer, B., Newman, K., Moore, M., Mayeux, P., Linder, D. (1963): The bacteriologic and serologic incidence of Leptospirosis among striped skunks in Louisiana. Z.R., 2: 13-38.

Roth, E.E., Greer, B., Moore, M., Newman, K., Sanford, G.E., Adams, W.V., (1964): Serologic analysis of two new related leptospiral serotypes isolated in Louisiana. Z.R., 3: 31-36.

Roth, E.E., Linder, D., Adams, B.S. (1961): The use of agar plates as an aid for the isolation of leptospires. Amer. J. Vet. Res., 22: 308-312.

Russell, C.M. (1956): A hemolysin association with leptospirae. J. Immunol., 77: 405-409.

Russell, R.R., Hansen, N.F. (1958): The incidence of *Leptospira hyos* and *Leptospira pomona* infections in pigs in New Zealand. N.Z. Vet. J., 6: 50-51.

Ryan, T.J. (1978): The epidemiology of *Leptospira pomona* in pigs. Unpub. Ph.D. Thesis, Massey University, Palmerston North.

Ryan, T.J., Marshall, R.B. (1976): Isolation of a leptospire belonging to serogroup Tarassovi. N.Z. Vet. J., 24: 212-213.

Ryu, E. (1965): The influencial conditions of kidney tissue on the isolation of leptospires. Bull. Inst. Zoo., Acad. Sinica, 4: 59-63.

Salisbury, R.M. (1954): Leptospirosis in New Zealand livestock. Roy. Sanit. Inst. J., 15: 2-12.

Salminen, A. (1956): Studies on the occurrence of various leptospirae types in Finland. Ann. Med. Exp. Fenn., 34: Suppl. 5.

Santa Rosa, C.A., Sulzer, C.R., Pestana de Castro, A.F. (1972): A new leptospirae serotype in the Bataviae group, isolated in Sao Paulo, Brazil. Amer. J. Vet. Res., 33: 1719-1721.

Santo Rosa, C.A., Sulzer, C.R., Giorgi, W., da Silva, A.S., Yanaguita, R.M., Lobao, A.O. (1975): Leptospirosis in wildlife in Brazil: Isolation of a new serotype in the Pyrogenes group. Amer. J. Vet. Res., 36: 1363-1365.

Schlossberger, H., Langbein, H. (1952): Uber tragungvon leptospira ict durch *Ornithodorus monbata*. Z. Immunforsch., 109: 366.

Schneiderman, A., Greene, M.R., McClune, L.E., Dunn, M.S. (1953): Nutrition of *Leptospira canicola*. III. Utilization of vitamins and amino acids. Proc. Soc. Exp. Biol. Med., 82: 53-56.

Schnurrenberger, P.R., Hanson, L.H., Martin, R.J. (1970): Leptospirosis: Long-term surveillance on an Illinois farm. Amer. J. Epidemiol., 92: 223-239.

Schricker, R.L., Hanson, L.E. (1961): Effect of cortisone on *Leptospira pomona* infection in guinea pigs. Amer. J. Vet. Res., 22: 580-585.

Schwabe, C.W., Reimann, H.P., Franti, C.E. (1977): 'Epidemiology in veterinary practice.' Lea and Febiger, Philadelphia.

Sebek, Z., Wallner, H., Sixl, W., Kaaserer, G., Valova, M. (1976): Leptospiral antibodies in domestic animals in Tyrol. Folia Parasitol. (Praha), 23: 15-23.

Semenova, L.P., Soloshenko, I.Z., Ananyin, N.V. (1965): Leptospira of the Hebdomadis group. Report III. Detection of *Leptospira sejroe balcanica* subtype in the Soviet Union. J. Microbiol. Epidemiol. Immunobiol., 42: 61-44.

Shenberg, E., Birnbaum, S., Rodrig, E., Torten, M. (1977): Dynamic changes in the epidemiology of canicola fever in Israel. Natural adaption of an established serotype to a new resevoir host. Amer. J. Epidemiol., 105: 42-48.

Shenberg, E., Linderbaum, I., Dikken, H., Torten, M. (1975):
Isolation of a 'saprophytic' leptospiral serotype
*andamana* from carrier rats in Israel. Trop. Geogr. Med.,
27: 395-398.

Shield, J. (1974): Leptospirosis in the West, too. Queens. J. Ag.,
100: 231-232.

Shortridge, E.H. (1960): Leptospiral infection in rats. N.Z. Med.
J., 59: 558.

Shotts, E.B. (1976): Laboratory diagnosis of leptospirosis. In
'Biology of parasitic Spirochetes'. Academic Press (Inc.),
New York.

Shotts, E.B., Andrews, C.L., Harvey, T.W. ( 1975): Leptospirosis
in selected wild mammals of the Florida panhandle and South-
Western Georgia. J. Amer. Vet. Med. Ass., 167: 587-589.

Shotts, E.B., Andrews, C.L., Sulzer, C., Greene, E. (1971):
Leptospirosis in cottontail and swamp rabbits of the
Mississipi Delta. J. Wildl. Dis., 7: 115-117.

Shotts, E.B., Hayes, F.E. (1970): Leptospiral antibodies in white-
tailed deer of the south-eastern United States. J. Wildl. Dis.,
6: 295-298.

Silva, I. (1975): Unpublished. Cited in Anon (1975).

Sladen, B.K., Borg, F.N. (1969): 'Biology and Populations'.
Elsevier Publishing Co. Inc., New York.

Sleight, S.D., Langham, R.F., Morten, R.L. (1960): Experimental
leptospirosis: The early pathogenesis of *Leptospira pomona*
infection in young swine. J. Infect. Dis., 106: 262-269.

Sleight, S.D., Williams, J.A. (1961): Transmission of bovine
leptospirosis. J. Amer. Vet. Med. Ass., 138: 151-152.

Smith, D.J.W., Brown, H.E., Tonge, J.I., Sinnamon, C.N., MacDonald,
V.M., Ross, C.J., Doherty, R.L. (1954): The serological
classification of 89 strains of Leptospirae from North
Queensland including five serotypes new to Australia.
Aust. Ann. Med., 3: 98-105.

Smith, M.J., Brown, B.K., Frith, H.J. (1969): Breeding of the brush-
tailed opossum, *Trichosurus vulpecula* (Kerr), in New South
Wales. C.S.I.R.O. Wildl. Res., 14: 181-193.

Smith, J.M.B. (1964): Some microbiological aspects of the short-
eared European hedgehog, *Erinaceus europaeus*, in New
Zealand. Unpub. Ph.D. Thesis. University of Otago, Dunedin.

Smith, J.M.B. (1965): Leptospires in New Zealand animals.N.Z. Vet. J.,
13: 136.

Smith, R.T. (1960): Immunity in infancy. Pediat. Clin. N. Amer., 7: 269-281.

Spurr, E.B. (1975): Assessment of an opossum control operation in the Otira River catchment 1975, by point distance estimates of pellet density. F.R.I., N.Z. Forest Service Protection Forestry Rep., No. 142 (unpub.). 15pp.

Stalheim, O.H.V. (1966a): Some aspects of leptospirosis control. Proc. U.S. Livestock. San. Ass., 69: 170-174.

Stalheim, O.H.V. (1966b): Leptospirae selection, growth and virulence in synthetic medium. J. Bact., 92: 946-957.

Stalheim, O.H.V. (1971): Virulent and avirulent leptospires: Biochemical activities and survival in blood. Amer. J. Vet. Res., 32: 843-849.

Stavitsky, A.B. (1945): Studies on the pathogenesis of leptospirosis. J. Infect. Dis., 76: 179-192.

Stoenner, H.G., Grimes, E.F., Thrailkill, F.B., Davis, E. (1958): Elimination of *Leptospira ballum* from a colony of swiss albino mice by use of chlortetracycline H Cl Amer. J. Trop. Med. Hyg., 7: 423-426.

Stuart, R.D. (1946): The preparation and use of a simple culture medium for leptospirae. J. Path. Bact., 58: 343-349

Stuart, R.D. (1956): The importance of urinary antibodies in the diagnosis of leptospirosis. Canad. J. Micro., 2: 288-297.

Sturdza, N., Safiresco, D. (1964): Sur la colorabilite des leptospires et des triponemes. Ach. Roum. Pathol. exp. Microbiol., 23: 927-938.

Sullivan, N.D. (1970): Experimental infection of cattle with *Leptospira hardjo*. Aust. Vet. J., 46: 121-122.

Sullivan, N.D. (1974): Leptospirosis in animals and man. Aust. Vet. J., 50: 216-222.

Sullivan, N.D., Callan, D.P. (1970): Isolation of *Leptospira hardjo* from cows with mastitis. Aust. Vet. J., 46: 537-539.

Sulzer, C.R., Glosser, J.W., Rogers, F., Jones, W.L., Frix, M. (1975): Evaluation of an indirect haemagglutination test for the diagnosis of human leptospirosis. J. Clin. Microbiol., 2: 218-221.

Sulzer, C.R., Harvey, T.W., Galton, M.M. (1968): Comparison of diagnostic technics for the detection of leptospirosis in rats. Hlth. Lab. Sci., 5: 112.-116.

Sulzer, C.R., Jones, W.L. (1974): Leptospirosis. Methods in laboratory diagnosis. U.S.D.H.E.W. Pub. No. (CDC) 76 - 8275, Atlanta, Georgia.

Sulzer, C.R., Shotts, E.B., Olsen, C.D., Galton, M.M., Stewart, M.A. (1964): Leptospirosis due to serotype *hardjo* in cattle. J. Amer. Vet. Med. Ass., 144: 888-890.

Sveshnikova, N.P., Chernukha, Y.G. (1972): To the question of the character of the variability of the antigenic structures of Leptospira. J. Hyg. Epidemiol. Microbiol. Immunol., 16: 346-351.

Tabel, H. (1970): Local production of antibodies to *Leptospira pomona* in kidneys of chronically-infected skunks (*Mephitis mephitis*). J. Wildl. Dis., 6: 299-304.

Tabel, H., Karstad, L. (1967): The renal carrier state of experimental *Leptospira pomona* infections in skunks (*Mephitis mephitis*). Amer. J. Epidemiol., 85: 9-16.

Tan, D.S.K., Welch, Q.B. (1974): Evaluation of *Leptospira biflexa* antigens for screening human sera by the microscopic agglutination (MA) test in comparison with the sensitised erythrocyte lysis (SEL) test. S.E. Asian J. Trop. Med. Pub. Hlth., 5: 12-16.

Taylor, P.L., Hanson, L.E., Simon, J. (1970): Serologic, pathologic and immunologic features of experimentally-induced leptospiral nephritis in dogs. Amer. J. Vet. Res., 31: 1033-1049.

Te Punga, W.A., Bishop, W.H. (1953): Bovine abortion caused by infection with *Leptospira pomona*. N.Z. Vet. J., 1: 143-149.

Thieler, K. (1972): 'The house mouse. Development and normal stages from fertilization to four weeks of age.' Springer-Verlag, Berlin, Heidelberg, New York.

Torten, M., Birnbaum, S., Klingberg, M.A., Shenberg, E. (1970): Epidemiologic investigation of an outbreak of leptospirosis in the Upper Galilee, Israel. Amer. J. Epidemiol., 91: 52-58.

Torten, M., Shenberg, E., van der Hoeden, J. (1965): The role of birds in the epidemiology of leptospirosis. Trop. Geogr. Med., 17: 353-358.

Trainer, D.O., Hanson, R.P., Pope, E.E., Carbrey, E.A. (1963): The role of deer in the epizootiology of leptospirosis in Wisconsin. Amer. J. Vet. Res., 24: 159-167.

Tripathy, D.N., Hanson, L.E. (1974): Pathogenicity of *Leptospira grippotyphosa* for gerbils, hamsters and voles. Amer. J. Vet. Res., 35: 547-549.

Tripathy, D.N., Hanson, L.E. (1976): Some observations on chronic leptospiral carrier state in gerbils experimentally infected with *Leptospira grippotyphosa*. J. Wildl. Dis., 12: 55-58.

Tripathy, D.N., Hanson, L.E., Mansfield, M.E. (1973): Growth inhibition test for measurements of immune response of animals vaccinated with leptospiral bacterins. Proc. U.S. An. Hlth. Ass., 77: 113-118.

Tripathy, D.N., Smith, A.R, Hanson, L.E. (1975): Immunoglobulins in cattle vaccinated with leptospiral bacterins. Amer. J. Vet. Res., 36: 1735-1736.

Troughton, E. (1946): 'Furred animals of Australia'. Angus and Robertson, Sydney.

Turner, L.H. (1967): Special Article.Leptospirosis. I. Trans. Roy. Soc. Trop. Med. Hyg., 61: 842-855.

Turner, L.H. (1968): Special article. Leptospirosis. II Serology. Trans. Roy. Soc. Trop. Med. Hyg., 62: 880-899.

Turner, L.H. (1970): Special article. Leptospirosis III. Maintenance, isolation and demonstration of leptospires. Trans. Roy. Soc. Trop. Med. Hyg., 64: 623-646.

Twigg, G.I., Cuerden, C.M., Hughes, D.M. (1968): Leptospirosis in British wild mammals. Sym. Zoo. Soc. Lond., 24: 75-98.

Twigg, G.I., Cuerden, C.M., Hughes, D.M., Medhurst, P. (1969): The leptospirosis resevoir in British wild mammals. Vet. Rec., 84: 424-426.

Twigg, G.I., Hughes, D.M., McDiarmid, A. (1972): Leptospiral antibodies in dairy cattle: Some ecological considerations. Vet. Rec., 90: 598-602.

Twigg, G.I., Hughes, D.M., McDiarmid, A. (1973): The low incidence of leptospirosis in British deer. Vet. Rec., 93: 98-100.

Tynedale-Biscoe, C.H. (1955): Observations on the reproduction and ecology of the brush-tailed opossum, *Trichosurus vulpecula* Kerr (Marsupialia) in New Zealand. Aust. J. Zoo., 3: 162-184.

Uhlenhuth, P., Fromme, W. (1930): Weilsche Krankheit. In Hanbuch der Pathol. Mikroorganismen, 7: 487. Cited by Babudieri (1958).

Uhr, J.W. (1964): The heterogencity of the immune response. Science, 145: 457-463.

Urquhart, A., Grant, L., Sulzer, C. (1973): A new leptospiral serotype belonging to serogroup Icterohaemorrhagiae. J. Wildl. Dis. 9: 254-257.

Valentine, B.L., Morter, R.L., Jackson, H.D. (1964): A hemolytic factor associated with *Leptospira pomona* cells. Proc. Soc. Exp. Biol. Med., 117: 810-813.

van der Hoeden, J. (1954): The pathogenicity of Leptospiras to field rodents in Israel (A new test animal for use in Leptospira research). J. Infect. Dis., 95: 213-219.

van der Hoeden, J. (1955): Epizootiology of leptospirosis *(canicola)* in the bovine and other species in Israel. J. Amer. Vet. Med. Ass., 122: 207.

van der Hoeden, J. (1958): Epizootiology of leptospirosis. Adv. Vet. Med., 4: 277-339.

van der Hoeden, J. (1964): Leptospirosis. In 'Zoonoses'. Elsevier Publishing Co., Amsterdam, London, New York.

van der Hoeden, J. Shenberg, E. (1962): Infections with *Leptospira mini, swazijak,* in man and animals in Israel. Z.R., 1: 25.

van der Hoeden, J., Shenberg, E., Evenchik, Z. (1961): Leptospira agglutinating factors in turtle sera. Nature, 190: 95-96.

van Theil, P.H. (1948): 'The Leptospiroses'. University of Leiden Press, Leiden.

Vedros, N.A., Smith, A.W., Schonewald, J., Migaki, G., Hubbard, R.C. (1971): Leptospirosis epizootic among California sea lions. Science, 172: 1250-1251.

Vervoot, H. (1922): Spirochaeten bij acute koortsige zicklen van onbekenden oorsprong in de Tropen. Geneesk. Tijdschr. Ned. Ind., 62: 692. Cited by Sulzer and Jones (1974).

Vysotskii, B.V., Ryashchenko, L.P. (1961): Leptospirosis in spotted deer *(Cervus nippon).* J. Microbiolol. Epidemiol. Immunobiol., 5: 67-78.

Watson, J.S. (1959): Identification of rats and mice in New Zealand. N.Z.J.Ag., 365-368.

Webster, W.M. (1957): The hedgehog as a potential resevoir of *Leptospira pomona.* N.Z. Vet. J., 5: 113.

Webster, W.M., Reynolds, B.A. (1955): Immunization against *Leptospira pomona.* N.Z. Vet. J., 3: 47-59.

White, F.H. (1963): Leptospiral agglutinins in snake serum. Amer. J. Vet. Res., 24: 179-182.

Willis, M.F., Wannan, I.S. (1966): Some aspects of the epidemiology of leptospirosis in New Guinea. Med. J. Aust. 14: 129-136.

Winter, J.W. (1963): Observations on a population of the brush-tailed opossum *(Trichosurus vulpecula* Kerr). Unpub. M.Sc. Thesis, University of Otago, Dunedin.

Wodzicki, K.A. (1950): 'Introduced mammals of New Zealand. An
        ecological and economic survey'. N.Z. D.S.I.R. Bulletin
        No. 98 , Wellington

Wolff, J.W. (1954): 'The laboratory diagnosis of leptospirosis.'
        Charles C Thomas, Springfield, Illinois.

Wolff, J.W. (1963): In Acta Leidensia, 32: 309-314. Cited by
        Turner (1967).

Wolf, J.W., Bohlander, H.J. (1965): Leptospiral infections of
        hedgehogs in the Netherlands. Trop. Geogr. Med., 1: 9-16.

Woods, G.T., Gustafson, D.P., Hanson, L.E., Alberts, J.O. (1962):
        Experimental infection of swine with *Leptospira ballum*.
        Z.R., 1: 165-184.

Yadav, M. (1971): The transmission of antibodies across the gut
        of pouch-young marsupials. Tmmunol., 21: 839-851.

Yager, R.H., Gochenour, W.S., Alexander, A.D., Wetmore, P.W. (1953):
        Natural occurrence of *Leptospira ballum* in rural house
        mice and an opossum. Proc. Soc. Exp. Biol. Med., 84: 589-590.

Young, B.J. (1969): A reliable method for demonstrating spirochaetes
        in tissue sections. J. Med. Lab. Technol., 26: 248-252.

Zwierz, J., Durlakow, I., Karmanska, K., Zwierzchowski, J., Lazuga, K.,
        Korczynska, A. (1958): Badanie fauny w ogniskach epidemii
        leptospirozy w powiercie Tomaszow Lubelski. Med. Weteryn-
        aryjna, 11: 647. Cited in Anon (1966).

APPENDIX I

Production of Antiserum

Antisera were produced in adult laboratory rabbits, according to the following procedure :

Day 1 : Heat-fix (30 minutes at $56^{\circ}C$) a seven day culture grown in liquid EMJH. The culture should contain $>10^{8}$ leptospires/ ml. Inoculate, via the ear vein, with 4 mls of culture.

Day 6 : Repeat procedure for Day 1.

Day 11 : Inoculate a seven-day, live culture ($>10^{8}$ leptospires/ml) via the ear vein.

Day 18 : Anaesthetise rabbit with ether and bleed by cardiac puncture.

Store antiserum at $-20^{\circ}C$.

APPENDIX II

## Preparation of EMJH medium (Difco)

The components of this medium consist of –

    a)  Bacto-Leptospira medium base EMJH

    b)  Bacto-Leptospira enrichment EMJH

These components are prepared according to the formulations described by Ellinghausen and McCullough (1965), as modified by Johnson and Harris (1967).

To rehydrate the medium, suspend 2.3 g in 900 ml of distilled water and agitate until completely dissolved. Autoclave for 15 minutes at 6.8 kg pressure ($121^{o}$C). Allow the sterile medium base to cool to room temperature. Aseptically add 100 ml of enrichment. Mix gently and then dispense as required.

For the preparation of semisolid medium (0.15%), add 1.5 g agar* to 900 mls of base medium, before autoclaving.

## Preparation of EMJH medium containing 5FU

Dissolve 1.0 g 5FU** in 50 ml of distilled water and then add 2.0 ml 2N NaOH and heat gently (<$56^{o}$C). Adjust pH of the solution to 7.5 with 1N HCl and bring the volume to 100 ml with distilled water. Sterilize the solution by 0.22μm membrane filtration. This is the stock solution, which is stored at $4^{o}$C. 2.0 ml stock solution added to 100 ml medium provides a final concentration of 200 μg 5FU/ml.

*  Difco, Detroit, Michigan, U.S.A.

** Sigma Chemical Co., P.O. Box 14508, St Louis, Missouri 63178, U.S.A.

APPENDIX III

## Calculation of geometric mean titre using coded titres

Titres are coded 0, 1, 2, 3, .... beginning with the lowest dilution of serum. The relationship between X, the titre expressed as a reciprocal dilution, and the coded titre, is

$$\text{coded titre} = \log\left(\frac{X}{C}\right)$$

where c = reciprocal dilution of the serum which is coded as 0; and the base of the logarithms is the same as the dilution factor (two).

The GMT is calculated by

    (a)  finding the arithmetic mean (M) of the coded titres

    (b)  GMT = (antilog of M) X C.

## Sensitivity and Specificity

Sensitivity is the probability of a diagnostic test correctly identifying as positive those animals which are truly positive.  Specificity is the probability of identifying as negative those animals that are truly negative.

True state of population

|  |  | + | - |  |
|---|---|---|---|---|
| Test result | + | N++ | N+- | N+. |
|  | - | N-+ | N-- | N-. |
|  |  | N.+ | N.- | N.. |

N.. = population size

$$\text{Sensitivity} = \frac{N++}{N.+}$$

$$\text{Specificity} = \frac{N--}{N.-}$$

$$\text{Probability of a false positive} = \frac{N+-}{N.-}$$

$$\text{Probability of a false negative} = \frac{N-+}{N.+}$$

APPENDIX IV

## Growth Inhibition Test

Inactive sera in a water bath at 56°C for 30 minutes. Following 0.22 μm membrane filtration of sera, aseptically put five, 0.05 ml replicates of each serum sample into sterile, screw-top culture tubes. Count test cultures in a Petroff-Hauser counting chamber and standardise to a density of 1 x $10^7$ leptospires/ml by the addition of liquid EMJH medium. Add 0.05 ml of culture to each serum sample and then add one ml of liquid medium. In addition to test sera, maintain positive controls (sera containing leptospiral titres) and negative controls (media and cultures only).

Incubate tubes at 30°C for seven days and then examine by dark-field microscopy for leptospiral growth.

APPENDIX V

## Preparation of Gel filtration Buffer (PBS)

| Chemical | Weight(g) |
|---|---|
| $Na_2HPo_4$ | 60 |
| $NaH_2Po_4 2H_2O$ | 12.5 |
| NaCl | 425 |

Dissolve in 5 L of deionized distilled water. Check that pH is 7.4 to 7.5. This is stock PBS (10 X concentration). To prepare working solution, take 100 mls of stock PBS and dilute to one litre with deionized, distilled water.

## Preparation of sodium barbital buffer

Dissolve 8.52 g of barbituric acid and 47.38 g barbitone sodium in 2.5 litres of distilled water. Dilute to 5 litres and adjust pH to 8.6 with 0.5 M HCl.