Diseases of the Eastern Barred Bandicoot (*Perameles gunnii*) with Special Reference to Toxoplasmosis and the Marsupial Immune System.

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by

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Thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy of the University of Tasmania

2000

Statement

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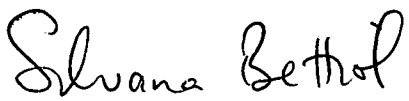

Silvana Bettiol

Part of the work contained in this thesis has been published, submitted for publication, or presented at local and international conferences as follows:

Bettiol SS, Obendorf DL, Nowakowski M, Goldsmid JM. (2000). Pathology of experimental toxoplasmosis in Eastern Barred Bandicoots. J Wildl Dis 36 (1): 141-144.

Bettiol SS, Obendorf DL, Nowakowski M, Milstein T, Goldsmid JM. (2000). Earthworms as paratenic hosts of toxoplasmosis in Eastern Barred Bandicoots. J Wildl Dis 36 (1): 145-148,

Kettlewell JS, **Bettiol SS,** Davies N, Milstein T and Goldsmid JM. (1998). Epidemiology of Giardiasis in Tasmania: A potential Risk to Residents and Visitors. J Travel Med 5 (3): 127-130.

Bettiol SS, Jakes K, Le DD, Goldsmid JM, and Hocking G. (1998). First record of trypanosomes in Tasmanian bandicoots. J Parasitol 84 (3): 538-541

Bettiol SS, Kettlewell JS, Davies NJ, and Goldsmid JM. (1997). Giardiasis in native marsupials of Tasmania. J Wildl Dis 33(2): 352-354.

Bettiol SS and Obendorf DL. (1997). Cats and Toxoplasmosis. Invertebrata: Tasmania's Invertebrate Newsletter. 9: 4.

Bettiol SS, Goldsmid JM, Le DD, and Driessen M. (1996). The first record of a member of the genus *Hepatozoon* in the Eastern Barred Bandicoot (*Perameles gunnii*) in Tasmania. J Parasitol. 82 (5): 829-830.

CONFERENCE PRESENTATIONS:

Bettiol SS and Goldsmid JM. (1999). Protozoan Infections of Marsupials in Tasmania. 26th World Veterinary Congress WVA, Mondial Vet Lyon, 99. Poster session: Wild Animal Health. Palais Des Congres Cite a Lyon - France, 23rd – 26th September.

Bettiol SS and Goldsmid JM. (1999). Toxoplasmosis in Tasmania. The Australian Society for Infectious Diseases Inc. and the Australasian College of Tropical Medicine. Annual Scientific Meeting, Cairns, Queensland, 17 – 21st April.

Bettiol SS, Goldsmid JM, Kettlewell J, Davies N, Paget S and Milstein T. (1997). *Giardia* Infections of Tasmanian Marsupials. Australasian Section, Wildlife Disease Association. Lady Barron, Flinders Island, 12-17th October.

Bettiol SS, Goldsmid JM, Kettlewell J, Davies N, Paget S and Milstein T. (1997). The Clinical Significance and Epidemiology of Giardiasis in Tasmania. Australasian College of Tropical Medicine, 6th Annual Scientific Meeting. New Images in Tropical Medicine. Townsville, Queensland, 19-20th July.

Bettiol SS and Goldsmid JM. Protozoan Infections of Tasmanian Marsupials. Proferred paper. (1997). Veterinary and General Parasitology. 10th International Congress of Protozoology, Sydney, NSW, 21-25th July.

Bettiol SS and Goldsmid JM. (1994). Parastitic Infections of the Eastern Barred Bandicoot. Proferred Paper. Zoonoses II. Australasian College of Tropical Medicine, 7th Scientific Meeting. Tropical Medicine at the Bottom End, Hobart, Tasmania, 25-27th November.

Abstract

The reasons for the population decline of the marsupial the Eastern Barred Bandicoot, *Perameles gunnii*, in Tasmania, and the probable role of their susceptibility to protozoan diseases such as *Toxoplasma gondii* remain elusive. The pathological hallmarks of the disease have been extensively investigated in other animal species and although some species are more vulnerable to toxoplasmosis than others, the reason for marsupial vulnerability remains to be fully defined.

This thesis has sought to address these and other factors that might be associated with the relationship between the marsupial, its microbial and parasitic diseases, ecological surroundings and immune system. Secondly, it was essential to examine the current disease status of *P. gunnii* in relation to its zoonotic potential in the Tasmanian setting. Finally, it was necessary to attempt to investigate the immunological status of this animal.

The major conclusions from these investigations were that *P. gunnii* is extremely susceptible to even low doses of *T. gondii* oocysts. The development of clinical disease is rapid, with high morbidity and mortality. The species appears to be susceptible to other Apicomplexan species including *Hepatozoon* sp., and the flagellate *Giardia*. The close association of *P. gunnii* with humans and the infringement of humans into their habitat makes this marsupial vulnerable to trauma, predation, shelter and food deprivation, and exacerbation of current parasitic and microbial diseases. In summary, this study further clarifies the relationship between humans, *P. gunnii* and parasite and the possible zoonotic potential that exists within Tasmania.

Acknowledgements

I would like to sincerely thank Professor John Goldsmid for providing me with the opportunity to accomplish this project, for his guidance, extensive knowledge and support. I wish to thank Dr Greg Woods for his unlimited patience, advice, assistance and computer skills. I would like to thank Prof H. Konrad Muller for the opportunity to undertake this degree within the Discipline of Pathology.

I also wish to thank the entire team of the Discipline of Pathology who, over the past years, have shared with me their technical support, enthusiasm and friendship. I would especially like to thank Dennis Quinn for his constant support during this project. Thanks to Mark Cozens for performing flow cytometry and Geoff Phillips for the histological work; Irene Jacobs for her work on the electron microscope; Dr Geoff Dandie for assistance in the animal house; Dr James Vickers for computer advice and assistance; Imogen Liew for assistance with the statistical analysis of data; Hilary Goldsmid for the wonderful artwork; Lorna Glasovac for encouragement, friendship, and support; Jacqui Robertson for support and interest in my work; Tal Milstein, Jenny Kettlewell, Debbie Orchard, Katie Jakes, Daniel Le and Anita John for their support and friendship.

I gratefully acknowledge Michael Driessen, Steven Mallick, and Greg Hocking from Parks and Wildlife Service for their assistance, especially during trapping sessions and specimen collections. Thanks to all other field officers and staff at Parks and Wildlife Service for advice during the field work.

Special thanks to Dr David Obendorf, Dr Tim McManus, Dr Barry Munday, Dr John Seebeck, and Dr Barry Wells for their advice and support throughout the

years. Thanks to Assoc. Professor Stewart Nichols and Dr Niels Andersen for their assistance. Thanks to Mark and Andrew Nowarkowski; and staff at the Royal Hobart Hospital who provided advice whenever it was sought; Jerry Veldhaus and Pat Statham from the Department of Primary Industry and Water Environment for technical advice.

I would like to thank David Jacobs for all the advice in handling captive marsupials at the start of this project. Thanks to Dr Eileen Wronski and staff at the Central Animal House for their advice and constant support; Julie Brassington for air lifting water out of the Pelion region in remote south-west Tasmania. Thanks to Dr R Shanker and to technical support staff at the Macquarie University for advice in water processing and flow cytometry. I would like to express my gratitude to Dr. JP Dubey of USDA, ARS Livestock & Poultry Sciences Institute (Beltsville, USA) who kindly supplied the *Toxoplasma gondii* oocysts.

Part of this work was supported by a Small Grant from the Australian Research Council and Heritage and Wildlife fund of the Tasmanian Parks and Wildlife Service.

Finally to all my friends who have put up with this PhD for such a long time, you certainly know who you are.

To my mum, dad, Angela, Sante and nephews.

Abbreviations

BSA	Bovine Serum Albumin
Con A	Concanavalin
CMI	Cell-mediated immunity
CPM	Counts per minute
DAT	Direct agglutination test
DPBS	Dulbecco's phosphate-buffered saline
DPIWE	Department of Primary Industry, Water and Environment
EBB	Eastern Barred Bandicoot
EDTA	Ethylenediamine tetra-acetic acid
ESA	Excreted-secreted antigens
FISF	Flinders Island Spotted Fever
Ig	Immunoglobulin
JAC	Jacalin
LL	Lectin
LPS	Lipopolysaccharide
MAT	Modified agglutination test
NBT	Nitroblue tetrazolium
NK	Natural Killer cells
OsO_4	Osmium tetroxide
PHA	Phytohaemagglutinin
PMA	Phorbol-myristate-acetate
PME	Postmortem examination
PMNC	Polymorphonuclear cell
PWM	Pokeweed mitogen
QSF	Queensland spotted fever
RR	Ross River virus
SEM	Scanning electron microscope
SI	Stimulation index
SRBC	Sheep red blood cell
TEM	Transmission electron microscope

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Chapter 1. Literature Review

1.1 INTRODUCTION

Historically the Marsupialia (Metatheria) have held great interest for comparative immunologists due to their unique primitive features and similarities with the Placentalia (Eutheria). The attraction of marsupials as experimental animals has principally been to explore the evolution of adaptive immunity in placentals, while the immature marsupial neonate has been regarded as an accessible model to study the ontogeny of immune competence (Jurd, 1994).

Considering the advances in the fields of comparative immunology, the role played by disease in the ecology of populations has often been overlooked. In recent years there has been a growing awareness of the effect of disease on biodiversity, the integrity of ecosystems and animal populations, both alone and in combination with habitat destruction, world climate changes, predation and humans. For many Australian marsupials this combination has resulted in dramatic declines in species distribution and status since the time of European settlement. One such species is the Eastern Barred Bandicoot, *Perameles gunnii*. Today this species is endangered in the Australian State of Victoria and is a threatened species on the island of Tasmania. Many factors now affect population numbers but the devastating impact of toxoplasmosis on this species reinforces the recognition of disease agents as an important starting point of disease surveillance of wildlife.

It is difficult to ignore the role of interacting factors when studying a threatened species or the interactions between a parasite, bacterium or virus with its host. Measuring the responses to the interactions between the organism and its host are complicated by all the interacting factors which may serve to confuse the issue (Gulland, 1995). Conversely, studying the interactions in isolation from other factors, or *in vitro*, is also an artificial situation. The scope of this investigation

focuses on the impact that infectious agents might have upon the future survival of the marsupial and encompasses the role of *P. gunnii* as a potential reservoir of parasitic, bacterial and viral agents which might infect humans.

It is appropriate here, to point out that while there appears to be a notable accumulation of data in regard to the taxonomy of this marsupial and related species, very little new work has focussed on the biology and immunology of *P. gunnii.* Where there is a void in the literature in this respect in regard to *P. gunnii*, other bandicoots or related marsupials are used as examples.

1.2 THE EASTERN BARRED BANDICOOT (PERAMELES *GUNNII*)

1.2.1 Taxonomy

The super-family Peramaloidea is the third and final major lineage of Australian marsupials which split from the rest of the Australian marsupials approximately 48 million years ago (Baverstock *et al.*, 1990). They are currently found in Australia, New Guinea and neighbouring islands. The taxonomic position of the bandicoot has puzzled many researchers as they share anatomical features linking them with two major groups of marsupials. One feature is the syndactylous toe, where the second and third toes on the hind foot are fused together with a skin layer at the base similar to the herbivorous or diprotodont marsupials, with free terminal joints and claws (Gordon, 1984; Schneider, 1990). The fourth digit is elongated and this syndactylous condition is believed to have evolved as a specialised structure, often functioning as a hair comb. The bandicoot also possesses more than one incisor on the upper and lower parts of the jaw, similar to the carnivorous or polyprotodont marsupials of the family Dasyuridae (Bensley, 1903; Strahan, 1983).

Groves and Flannery, (1990) revised the families and general taxonomy of the bandicoots using skull characteristics, dividing them into two clearly recognisable

divisions at the family level; the Peramelidae, an open country bandicoot and the Peroryctidae comprising the tropical rainforest bandicoots (Appendix 11.1). Skulls of the Peramelidae are characteristically flat, whereas those of the Peroryctidae are cylindrical. The latter have a much higher cranium with subcylindrical rostral and neurocranial portions in cross section. A symposium on bandicoots and bilbies organised by the Australian Mammal Society, adopted this revision (Seebeck *et al.*, 1990), while recognising the work of Baverstock *et al.*, (1990) who further separated the Perameloidea into three lineages based on albumin immunology. Whole-serum serology (Kirsch, 1968, 1977; Lowenstein *et al.*, 1981), and albumin immunological distance data (Maxon *et al.*, 1975) supports the work of Baverstock *et al.*, (1990) indicating that the bandicoots are the sister-group to the dasyurids. The work of Thomas *et al.*, (1989) on DNA sequencing, supported a closer relationship between bandicoots and diprotodontians but Faith, (1990) argues the data shows no significant difference than that expected by chance. While controversy continues, more DNA hybridization data from Kirsch *et al.*, (1991) has suggested that the Perameloidea may lie outside the Australian radiation entirely.

Divisions within the bandicoot group itself are also constantly being reviewed. Since 1977, two families have been recognised: the Thylacomyidae (Archer and Kirsch, 1977) containing the Recent *Macrotis* and the Tertiary *Ischnodon australis* Stirton, and the family Peramelidae (Muirhead and Filan, 1995). More recently, the order Peramelemorphia has been divided into the Peramelidae and Peroryctidae. Peramelidae contain the predominantly Australian taxa *Perameles, Isoodon, Macrotis* and presumably *Ischnodon* (Muirhead and Filan, 1995), while the Peroryctidae contain the predominantly New Guinean taxa of *Echymipera, Peroryctes, Mircorperoryctes*, and *Rhynchomeles* (Groves and Flannery, 1990). The status of *Macrotis* remains undecided (Baverstock *et al.*, 1990; Muirhead and Filan, 1995).

Within the Australian taxa the short-nosed bandicoots (*Isoodon*) comprise three species, *Isoodon obesulus*, *Isoodon macrourus* and *Isoodon auratus*. A fourth species may also exist, with a new bandicoot recently found in a small pocket of forest in northern Australia (Anon, 2000). There are four existing species of the long-nosed (*Perameles*) bandicoot, *Perameles gunnii* (Eastern Barred Bandicoot), *Perameles nasuta* and *Perameles bougainville* (George *et al.*, 1990). *Perameles nasuta* and *Perameles bougainville* occur in the east and west regions of the Australian mainland respectively (Groves and Flannery, 1990). A fourth species, the desert bandicoot (*Perameles eremiana*) is presumed extinct (George *et al.*, 1990). There are two species of bandicoots found in Tasmania, the Eastern Barred Bandicoot (*P. gunnii*) and the Southern Brown Bandicoot (*Isoodon obesulus*) (Hocking, 1990).

1.2.2 General Description

The Peramelids are described as stocky and thickset in build with short limbs and neck, a long pointed muzzle, with insectivore teeth and powerful fore-claws for digging (Gordon *et al.*, 1989). The forelegs of *P. gunnii* are shorter than the hind legs, and are used for digging and extracting soil invertebrates which are their main food source. *P. gunnii* have large prominent ears, unlike the *Isoodon* spp., in which the ears are more rounded. Gordon *et al.*, (1989) correlated this elongation with the use and habitation of more open habitats. The tail is short and non-prehensile with little function in locomotion. *P. gunnii* and *I. obesulus* both have the ability to jump into the air while moving and immediately initiate rapid movement in another direction upon landing (Moloney, 1982; Coulson, 1990). *P. gunnii* have soft, grizzled brown-grey fur with three to four characteristic pale transverse stripes on the dorsal region. The ventral region, parts of the feet and tail are of a creamy white colour (Driessen *et al.*, 1991). Gordon *et al.*, (1989) described all Australian bandicoot species as having a dark pelage on the dorsal and lateral sides, with a white ventral region.

1.2.2.1 Diet

P. gunnii are nocturnal terrestrial marsupials which use their sense of smell to find food while hunting at twilight or during the night (Seebeck *et al.,* 1990). Their diet is omnivorous and possibly opportunistic. Although the bandicoot is considered an omnivore, feeding on insects as well as eating grass and fruit, they have been noted to be occasional opportunistic carnivores scavenging from carcasses when available (Goldsmid, personal communication).

In the wild, moisture obtained from their diet and from dew appears to be sufficient although in captivity *P. gunnii* are noted to drink water. Heinsohn, (1966), analysed the diet of *P. gunnii* and listed a range of soil-dwelling and surface invertebrates. The diet included earthworms (Lumbricidae), black crickets (*Gryllus servellei*) moths (Lepidoptera) and cockchafer grubs (Coleoptera; Scarabaeidae) as well as assorted large insect larvae (Phalaenidae, Hepialidae, and Elateridae) (Seebeck, 1979; Robinson *et al.*, 1991), small slugs (probably Limacidae), snails and small lizards (Lyne, 1964). They have been described as consuming a variety of plant material perhaps when insects are unavailable, including fruits, berries (*Rubus fruticosus*), grass, fungi and seeds (Smith, 1992). The activities of several disaccharidases in the mucosa of the small intestine in a number of marsupials including *P. nasuta* and *I. obesulus,* have been evaluated and a significantly high activity level of trehalose and cellobiose recorded (Moloney, 1982). The disaccharide trehalose is found only in insects, while cellobiose is formed during the catabolism of cellulose confirming the ability of bandicoots to utilise sugars of both insect and plant origin.

1.2.2.2 Behaviour

Studies of free-ranging *P. gunnii* in Tasmania and Victoria, by Heinsohn ., (1966) and Dufty, (1991), respectively, observed foraging and grooming to be the most

frequent activities of these animals. The home range of *P. gunnii* is variable, with females covering an area of approximately 2.2ha, while males cover areas ten times this size at around of 25.7 ha (Sherwin *et al.*, 1990). Bandicoots are often regarded as solitary and pugnacious, and the only social interactions seen are during reproductive activities or between mother and offspring (Stodart, 1966; Murphy, 1993). It appears that juveniles do not associate with their mothers for long periods (Lee *et al.*, 1985). The absence of parent-offspring association, and observations of dispersal by Heinsohn, (1966) suggest that a high level of juveniles are dispersed throughout bandicoot populations. Heinsohn, (1966) reported that the reaction of captive male and females was generally compatible, though in some instances the interactions are often those of mutual avoidance, or aggressive attacks that may lead to injury (Moloney, 1982).

1.2.2.3 Distribution

Rounsevell *et al.*, (1991) presented a detailed record of the distribution of native terrestrial mammals in Tasmania. In this comprehensive work, *P. gunnii* was primarily located in grassy woodlands and cleared pastoral land. The species was frequently recorded in the high rainfall regions of the north and south-east areas of Tasmania. Bandicoot numbers are now particularly low and fragmented across the drier areas of the Midlands and east coast (Mallick *et al.*, 1997a). There are no records of this species from the southwest region of the island, or from alpine areas above 950 metres. This includes rainforest areas of Tasmania, the alpine areas of the Central Plateau and areas of sedgeland vegetation. The species is commonly found on the Tasman Peninsula (Hocking, 1990; Rounsevell *et al.*, 1991). Rounsevell *et al.*, (1991) indicated that the *P. gunnii* was originally absent from all islands off the coast of Tasmania but was introduced to Bruny Island and Maria Island. Hope, (1973) reported fossil evidence suggesting that they inhabited the islands of Bass Strait during the Pleistocene age, at a time when these islands were linked to the mainland (cited by Hocking, 1990).

In Tasmania, *P. gunnii* and *I. obesulus* occur sympatrically and are distributed in eucalypt-dominated forest communities, natural grasslands and areas of cleared land (Seebeck, 1979; Hocking, 1990). Heinsohn, (1966) recorded nesting sites in hedgerows or scrub adjoining agricultural lands and urban areas. In contrast, Dufty, (1994) found that *P. gunnii* in Victoria use weedy habitats preferably along road sides and creek edges for nesting sites.

1.2.2.4 Reproduction

Heinsohn, (1966) described the gestation period of wild *P. gunnii* as short with the breeding season usually beginning in June and ending in February, while breeding season in captive species peaks from July to November (Seebeck, 1979). Heinsohn's, (1966) work in north-west Tasmania related the drop in fecundity near the end of the breeding season to climatic conditions and a decrease in the availability of food (Seebeck, 1979). Factors which initiate the breeding season still remain unclear.

The mean litter size of *P. gunnii* is approximately 2.3, with an average female producing 3.8 litters in the breeding season (Gemmell *et al.*, 1984). In contrast, a study by Stoddart and Braithwaite, (1979), found that *I. obesulus* produces 2.5 litters per female per year during a similar breeding season. There is a known variation in litter size amongst the same species of bandicoots in different locations and captive colonies (Gemmell *et al.*, 1984). For example, the breeding pattern of *I. macrourus* in New South Wales is interrupted during certain months but in Queensland this species breeds all year round (Smith, 1992).

The reproductive features of bandicoots makes the family Peramelidae unique amongst the marsupials (Gemmell *et al.*, 1984; Lyne, 1990). The animal's pouch opens downwards and outwards and contains eight teats arranged in two curved rows of four. As is typical for marsupials, the young are born in a rudimentary state and

are sheltered in the pouch throughout their later development. One of the unique characteristics of bandicoots is the structural and endocrinological feature required for further uterine foetal development. All bandicoots are polyoestrus and ovulation is spontaneous unlike most marsupials when oestrus precedes ovulation by one or two days (Tyndale-Biscoe *et al.*, 1987; Smith, 1992). The average length of an oestrus cycle can vary amongst bandicoots, but is approximately 21 days (Smith, 1992). Two species of bandicoots, *P. nasuta* and *I. macrourus* have a 12 day gestation period which is the shortest known among mammals, and is considerably shorter than their oestrous cycle.

While most marsupials utilise a yolk-sac placentation, bandicoots are the only marsupials to possess chorio-allantoic placentas similar to those of eutherian mammals. This structure was first described by Hill, (1897) in *I. obesulus* and later by Flynn, (1923) in *P. gunnii* and *P. nasuta* (cited by Seebeck, 1990). The placenta is very similar to that of the eutherian, except in regard to the trophoblast where it acts as an outer foetal boundary of the chorio-allantoic placenta and foetal component of the yolksac placenta (Padykula *et al.*, 1976; 1978; Smith, 1992). Cox, (1977) suggested that it may act as a protective barrier between foetus and mother against an immunological attack. The trophoblast does not persist as part of the placenta but disappears in late gestation and after eight days, the placenta is resorbed. Bandicoots also possess corpora lutea at pregnancy which persist into lactation (Gemmell, 1984). In Peramelids, the number of corpora lutea generally corresponds with the initial number of pouch young (Lyne *et al.*, 1976). The post-natal development of the bandicoot is rapid and neonates quickly reach independence and maturity as weaning occurs at about two months of age (Lyne, 1976). The loss of pouch young in captive bandicoots is reportedly high, possibly due to a natural reduction in the number of young that survive to weaning (Gemmell *et al.*, 1984; Gemmell, 1982). The survival rate of bandicoot young in the wild is unknown for most species.

The period of lactation in bandicoots is the shortest for any marsupial of comparable size. Suckling of young lasts only 55-60 days and during this time oestrus may occur resulting in the birth of a new litter once the young have vacated the pouch (Gemmell, 1982). During this period there is a five-fold increase in milk solids at peak lactation. Overall there is a tremendous energy demand by the young on the female bandicoot.

1.2.3 *P. gunnii* Ecology

Prior to settlement, *P. gunnii* were threatened by climate, fire and predation (Seebeck *et al.*, 1990). A vast number of potential causes have been examined in an attempt to elucidate specific factor(s) responsible for their recent decline, but this has proved difficult. Seebeck *et al.*, (1990) described the threat of disease, predation by introduced animals, loss of habitat and major alterations by agricultural advances as the obvious contributors to the loss of *P. gunnii* in Victoria. In Tasmania, *P. gunnii* are restricted to some privately owned land or, to Crown Land and reserves, and even though populations have managed to survive they remain vulnerable to changes in land management practices and urban expansion, as well as to disease and predation.

1.2.3.1 Life History

Since European settlement, the bandicoot is considered to have suffered more than any other Australian marsupial, resulting in a major decline in diversity and number, with "*P. gunnii* a notable example" Lyne, (1990). At least 12 Australian species of bandicoots have been described, with almost half now either extinct, rare, or threatened with extinction.

P. gunnii was once widespread across the south-west of Victoria (Seebeck, 1990), the southern and western regions of South Australia (Kemper, 1990), and numerous throughout the east and northern parts of Tasmania (Gould, 1863; Robinson *et al.*, 1993). European settlement has certainly been unkind to *P. gunnii* on the mainland,

with the species presumed extinct in South Australia and a once healthy Victorian population now reduced to a small remnant population near the city of Hamilton. In the past decade this population has continued to decline rapidly with an estimate of 441 animals / 1400 ha in 1985 by Sherwin *et al.*, (1990), to less than 200 animals / 2500ha by Seebeck *et al.*, (1990) and Dufty, (1991). Recently, the Victorian Department of Conservation and Environment instigated a breeding program at Gellibrand Hill Park in Victoria, with some success. Despite the apparent abundance of *P. gunnii* in Tasmania, it is currently listed as a vulnerable species, in recognition of the uncertain conservation status of the species in this State (ANZECC, 1991; Maxwell *et al.*, 1997).

1.2.3.2 Existing *P.* gunnii Populations in Tasmania and Victoria

Despite the larger population in Tasmania, investigations by Robinson *et al.*, (1993) found the genetic variability to be higher among mainland *P. gunnii* than in Tasmanian populations (higher average percentage differences (APDs), number of alleles, and heterozygosity at one locus). The absence of detectable mitochondrial DNA variability for restriction fragment patterns within Tasmanian populations was a surprising result considering the variability of a smaller sampled area, and fewer individuals from the area of Hamilton in Victoria. Robinson *et al.*, (1993) suggested three general scenarios may have lowered the genetic variability in Tasmania. The most likely was a single, or a few matriarchal lineages may have founded the existing populations. Secondly, the modification of the species habitat attributed to the recent fragmented population into isolated habitat pockets contributed to the low variability. Thirdly, mitochondrial DNA sequence divergence indicates a restriction or absence of gene flow between north and south sampled localities in Tasmania. This lack of genetic variability might pose severe survival problems in the Tasmanian *P. gunnii.*

1.2.3.3 Introduced Animals

The introduction of "non-native" animals to the Australian continent has directly and inadvertently had a profound impact on the survival of *P. gunnii.* The impact of the rabbit (*Oryctolagus cuniculus*) on Australia's landscape has been well documented and has contributed to the changing status of the bandicoot in numerous ways (Seebeck *et al.*, 1990). These influences include destruction of vegetation, the increase in the fox population in, and around Victoria, and by the use of artificial control measures such as poisoning (Brown, 1989 cited by Seebeck *et al*, 1990). Foxes (*Vules vulpes*) have been the most successful predator of bandicoots in Victoria, and their absence in Tasmania is considered advantageous for the existing *P. gunnii* population (Seebeck *et al.*, 1990). However, predation by domestic and feral cats (*Felis catus*) and dogs (*Canis familiaris*) are still regarded as significant causes of mortality in Tasmanian populations (Heinsohn, 1966). Both feral and domestic cats are described as opportunistic predators, capable of exploiting a wide range of prey depending on local availability (Jones and Coman, 1981).

The predatory habits of domestic cats and dogs in Tasmania were recently investigated using a survey of pet owners (Mallick *et al.*, 1997b). The results concluded that there was a tendency for both cats and dogs to prey on a broad range of species including arthropods, frogs, reptiles, exotic and native birds and mammals, with European rabbits and house mice identified as the main prey items. Cats were identified as the main predators of native bird species while dogs preyed mainly on native species of mammals. *P. gunnii* were taken by both pet types, with cats taking mainly juveniles, and dogs taking adult individuals. Mallick *et al.*, (1997b) considered these animals to pose a serious threat to native marsupial populations, particularly those rendered vulnerable by other factors such as fragmentation of habitat.

Unfortunately, feral cats harbour numerous protozoan and viral infections as well as helminth parasites and arthropods (Coman *et al.*, 1981a; 1981b) which perpetuate in the environment. The full impact of many of these diseases on native animals is not entirely known. The extreme vulnerability of marsupials when brought into contact with pathogenic protozoa such *T. gondii* for the first time may result from the fact that the marsupials evolved in the absence of the protozoa due to the geographical isolation of Australia and the relatively recent introduction of felines to the continent (Innes, 1997; Wagner, 1997).

1.2.3.4 Habitat

Major ecological changes experienced by both Tasmania and Victoria have included a reduction in native grasses resulting in new diversity and composition of grasslands in *P. gunnii* habitat. The changes in the physical structure of the grasslands have resulted in a reduction in availability of food resources, shelter and protection for *P. gunnii* (Seebeck *et al.*, 1990). Major changes in soil structure and soil invertebrate fauna have also been observed in what was considered *P. gunnii* areas by Brown, (1989), and which are now predominantly farmland holding stock (Seebeck *et al.*, 1990). Furthermore, the continued use of agricultural pesticides on the land was linked to high levels of organochlorine in bandicoot tissues. These levels have immediate consequences on individuals affecting their health, reproductive ability and the survival of their young (Seebeck *et al.*, 1990).

Expansion of residential areas into the natural habitat of *P. gunnii* has maximized the stress factor on the population. Urban sprawl has disturbed and reduced the natural habitat of *P. gunnii*, increased their confrontation with companion animals such as the cat and dog, and has increased the incidence of road kills (Mallick *et al.*, 1998). Recently, feasible management practices have been implemented to control and monitor these problems in both Victoria and Tasmania (Seebeck *et al.*, 1990).

1.3 BANDICOOTS AND THEIR DISEASES

1.3.1 Common Pathogens of the Bandicoot

Lenghaus *et al.*, (1990) reported that severe parasitic infections were common in free-ranging juvenile and adult *P. gunnii* of Victoria, when examined at postmortem. Heavy infestations of ticks, fleas, mites, lice and intestinal roundworms were often observed. Infections with the protozoan *T. gondii* were present in 10% of bandicoots investigated, often in association with other infections. Most of the common diseases recorded in captive marsupials also occur in the wild, mainly during times of great stress, such as drought or flooding when large numbers of individuals are gathered in small areas. Disease may also play a significant role in the ecology of rare and endangered species (Jacob-Hoff and Dunsmore, 1983; Speare *et al.*, 1989). A profile of recorded diseases has important implications for species conservation and management and a wide variety of such diseases have been recorded in both wild and captive *P. gunnii* (Lenghaus *et al.*, 1990). Many aspects of bandicoot ecology, including population-limiting factors are, unfortunately, often conducive to the occurrence of diseases in these animals.

Many marsupials are reservoir hosts to zoonotic and potentially zoonotic infections and in some instances both humans and animals are equally suitable reservoir hosts, with transmission occurring in both directions. With control of the domestic and companion animal-human infections, and with human expansion into remote areas, more contact is made with native animals. The emergence of a number of previously unreported viral, rickettsial, bacterial, and parasitic diseases has occurred in Tasmania over the last decade or more (Davies, 1995a). Diseases once thought to occur only on mainland Australia have been reported with increasing frequency, and new infections have been identified with the potential for others to be recognised as more research is carried out.

1.3.2 Viruses

Macropod herpesvirus (MHV)/Parma wallaby herpesvirus (PWHV) have been reported as causing disease and multiple mortalities amongst captive marsupials (Finnie *et al.*, 1976; Munday, 1988). Serological evidence has shown that antibodies to this or similar herpesviruses exist in wild macropods (Webber and Whalley, 1978). Although no outbreak of herpes virus has been reported in bandicoots, two genera in the family Peramelidae have been reported with antibodies to these viruses (Finnie, 1980). Lenghaus *et al.*, (1990) highlighted the fact these viruses may be in latent form but can be activated by stress such as captivity, overcrowding, nutritional deficiencies and inter- or intraspecific aggression. Together they can cause clinical and often, fatal disease.

Vale *et al.*, (1991) recorded serological evidence of arbovirus infection in native and domesticated mammals on the south coast of New South Wales. The study tested twelve native species and five introduced mammal species for antibodies to a variety of arboviruses. These included Ross River virus (RR), Barmah Forest virus (BF), Gan Gan virus (GG) and Trubanaman virus (TRU). RR virus is a well recognised cause of human epidemic polyarthritis (Fraser, 1986) and a range of vertebrates are susceptible to RR virus infection, with evidence suggesting that mammals are involved in the primary cycle (McManus and Marshall, 1986; Vale *et al.*, 1991). The major vertebrate hosts of RR virus are believed to be macropod marsupials though serological surveys have demonstrated the occurrence of infection in a wide range of wild and domestic animals (Aldred *et al.*, 1990; Vale *et al.*, 1991). Horses have often been considered to act as amplifier hosts in semi-urban areas (Gard *et al.*, 1977; Cloonan *et al.*, 1982). Although Cheng *et al.*, (1995) described RR virus as producing clinical symptoms only in humans and mice, there are reports of horses presenting with symptoms (Hueston, personal communication). Grey Kangaroos and Agile Wallabies have been reported developing viraemia from infection with RR virus with no ill effects after infection (Kay *et al.*, 1986 cited by Speare *et al.*, 1989).

Recent investigations by Ryan *et al.*, (1997) strongly indicate that fruit bats may act as vertebrate hosts and good dispersers in some areas.

Vale *et al.*, (1991) investigated bandicoot species including *I. obesulus* and *P. nasuta* and found seropositive animals (25% of bandicoots sampled) with RR virus titres ranging from 32 to 1024. This suggested that the Peramelidae were important potential reservoirs of RR virus given their widespread occurrence throughout the coastal areas of eastern Australia, northern Queensland and Tasmania.

The first evidence of RR virus in Tasmania was in the summer of 1974/75 in sentinel cattle in the north of the state (McManus and Marshall, 1986). Two of the three principal vector mosquitoes of mainland Australia were known to be absent from Tasmania but thorough investigation by McManus and Marshall, (1986) identified two mosquitoes known to be vectors of RR virus, in Tasmania, *Aedes flavifrons* and *Aedes camptorhynchus*. Since then an increasing number of human cases has been reported with the virus in Tasmania, the prevalence varying according to seasonal conditions and strategic rainfall necessary to support the vector mosquitoes. No serological survey has been performed on bandicoots in Tasmania to date.

1.3.3.1 Salmonellosis and Other Bacteria

A high proportion of apparently healthy captive macropods and other marsupials carry salmonellae in their gastro-intestinal tract or mesenteric lymph nodes (Atkinson *et al.*, 1953; Iveson and Bradshaw, 1973). Further, a wide variety of non-host-specific salmonellae have been isolated from macropod or kangaroo meat (Samuel, 1982; Speare *et al.*, 1989). For the most part, these bacteria are of concern only as a possible source of infection for humans (Samuel, 1982; Speare *et al.*, 1989). The disease spectrum caused by salmonellae is broad and can be characterised as primary salmonellosis, secondary salmonellosis, or asymptomatic salmonellosis. The course of salmonellosis varies between host species and is influenced by factors such as

stress and concurrent diseases. Stress has often been suggested to be the primary cause of susceptibility in both domestic and native animals including macropods and quokkas, and may be a problem in hand-reared joeys, causing gastroenteritis, septicaemia and frequently death (Barker *et al.*, 1963; Speare and Thomas, 1988).

The incidence of food-borne and enteric bacterial diseases amongst humans in Tasmania and mainland Australia reveals salmonellae as a major gastrointestinal pathogen. In Tasmania human salmonellosis is mainly due to *Salmonella* Typhimurium with increasing numbers of infections due to *Salmonella* Mississippi (de Boer, 1996). A study of native animals in Tasmania by Ball, (1991) revealed that a majority frequently excreted a wide variety of *Salmonella* serovars. Animals, in particular farm animals, have been regarded as major reservoirs of the salmonellae, with *Salmonella* Mississippi, in particular, being isolated from a range of animals, including, cats, dogs, goats as well as native species. The first report of salmonellae amongst bandicoots was by Lee and Mackerras, (1955). Two *Salmonella* spp., were identified in animals that had died in captivity. These included *Salmonella* Anatum in a black-striped wallaby and *S.* Lexington isolated from *Isoodon obesulus.* Amongst the Tasmanian native species, bandicoots, wombats, pademelons, wallabies, possums, Tasmanian Devils, quolls, ravens, tiger snakes, lizards and skinks have been reported as a source of *Salmonella* Mississippi (Anon., 1990; Davies, 1995a). A particularly high prevalence of *Salmonella* serovars have been reported in cold-blooded reptiles and skinks in several Australian surveys (Iveson *et al.*, 1969; Iveson and Hart, 1983; Ball, 1991).

The largest native Tasmanian carnivore, the Tasmanian Devil (*Sarcophilus harrisii*), of all native Tasmanian animals was reported to excrete the largest variety of *Salmonella* serovars, of which 48 % were *S.* Mississippi (Ball, 1991). Overall the carnivorous animals are more likely to encounter salmonellae than herbivores but the omnivorous *P. gunnii* whose main food interests are ground dwelling insects,

encounter a similar high risk of infection. Josland, (1951) (cited by Ball, 1991), found that salmonellae can persist for several months in faeces and soil, raising the possibility of coprophagic insects, such as earthworms, moving buried faeces to the surface and contaminating soil surfaces. This exposes digging animals such as *P. gunnii*, to bacterial pathogens. Oral ingestion is probably the most important mode of infection, although other routes including direct inoculation into the blood by ticks and possibly fleas, have been reported (Kramer, 1963).

Septicaemia in captive *P. gunnii* has been reported following injury and stress or malnutrition (Booth and McCracken, 1995). The most common reported organisms causing septicaemia were *Morganella morganii*, *Vibrio metschnikovii*, *Staphylococcus aureus*, *Escherichia coli* and *Proteus* sp. Lenghaus *et al.*, (1990) isolated several opportunistic bacterial pathogens from *P. gunnii* with severe exudative dermatitis, conjunctivitis and pouch infection. Miscellaneous opportunistic bacteria may be cultured as pure or mixed isolates from marsupials with liver abscesses, pneumonia or septicaemia (Munday, 1988; Speare, 1988; Bourne, 1997).

1.3.3.2 Rickettsiae

In Australia, the Spotted Fevers are the main diseases transmitted by ixodid ticks to humans. There are two presently recognised types of tick-borne Spotted Fever in Australia, Queensland Tick Typhus (QTT) or Queensland Spotted Fever (QSF) and Flinders Island Spotted Fever (FISF) (Russell, 1998). These afflictions are caused by rickettsiae (*Rickettsia australis* and *Rickettsia honei*, respectively).

FISF occurs on Flinders Island while an unknown rickettsial disease is present in northern Tasmania (Stewart, 1991; Russell, 1998; Graves, personal communication). The reptile tick *Aponomma hydrosauri* (Denny) has been implicated in one human case (Graves *et al.*, 1993) and it is believed that the blue tongue lizard may serve as

the reservoir host for *R. honei*. It also appears from DNA homology that *R. honei* is more closely related to the Thai tick typhus (TT-118) rickettsia than to any other member of the spotted fever group in Australia (Stenos *et al.*, 1998).

Queensland Tick Typhus is distributed along the eastern coastal strip of Australia from northern Queensland through northern New South Wales and as far south as Victoria (Graves, personal communication). It appears that the spotted fevers are transmitted to humans by the paralysis tick, *Ixodes holocyclus*, but other species such as *I. tasmanii* are also thought to be involved. The hard ticks, *Ixodes holocyclus*, *I. cornuatus* Roberts and *I. tasmanii* Neumann are suggested or confirmed vectors of *Rickettsia australis*, with native rats and bandicoots implicated as the reservoir host animals (Russell, 1998).

1.3.3.3 Spirochaetes

Lyme disease is a tick-borne infection with numerous manifestations in humans. The causative agent of Lyme disease is the spirochete *Borrelia burgdorferi* (Burgdorfer *et al.*, 1991) and other variant species (Benenson, 1995). Lyme disease can infect a wide range of domestic and wild animals producing a chronic arthritis in dogs, cattle, horses and wild deer. The transmission also to animals is via tick bites, with recent evidence of transmission by oral ingestion and transplacental infection (Munro, 1992). It has become the most frequently reported human tick-borne infection worldwide, although doubt remains as to whether it occurs in the southern hemisphere in general, including Australia (Russell, 1998). The main reason being the species of ticks shown to be competent vectors of *B. burgdorferi* to humans belong to the *Ixodes persulcatus* complex, and no species of this complex exist in Australia (Russell, 1998). A seabird tick, *Ixodes uriae* White, commonly associated with Lyme disease in Scandinavia, has been recorded from coastal and off-shore island sites in Australia imported with migratory birds (Hudson *et al.*, 1998).

The first Australian cases of a syndrome consistent with Lyme disease were reported from the Hunter Valley region of New South Wales in 1982 (Stewart *et al.*, 1982; Lawrence *et al.*, 1986). In Queensland and the eastern coast of NSW, the common bush tick *Ixodes holocyclus*, is well known from a range of native marsupials. Bandicoots are prone to heavy infestations of ticks and are important animals in the epizootic cycles (Munro, 1992). It was suggested by Munro, (1992) that bandicoots are a potential reservoir for Lyme disease in these regions. No cases of Lyme disease have been reported in Tasmania, but the potential threat of other tick-borne diseases such as Flinders Island Spotted Fever does exist.

In addition, *Leptospira*, a fragile spirochaetal bacterium, has been reported from a wide range of hosts, though clinical disease is uncommon. Prevalence of positive antibody titres to various serovars has been recorded in Tasmanian free-ranging Macropodidae including *Potorous tridactylus* (Munday, 1972a) and *M. rufogriseus rufogriseus* (Durfee and Presidente, 1979). Positive serology has also been confirmed in the long-nosed bandicoot (*Perameles nasuta*) and brown bandicoot (*Isoodon obesulus*) of south-eastern Australia, but no records from Tasmanian bandicoot species have been published

1.3.4 Fungal Diseases

Candida albicans infection is often reported as a problem with hand-reared marsupials (Bourne, 1997). Oral candidiasis has been reported in wild *P. gunnii* species at post-mortem (Obendorf, personal communication). Lenghaus *et al.*, (1990) reported severe *C. albicans* infection in hand-reared juvenile *P. gunnii.* Histologically the lesions were characterised by tissue necrosis and fluid exudation. The fungus is reported to occasionally lead to mucosal ulceration of the stomach with possible lesions of the liver, concurrent with necrotic gastro-enteritis (Speare, 1988; Canfield and Hartley, 1992). Diet and management stress is believed responsible for compromising the animals' immune system. Obendorf, (1980) described young

eastern grey kangaroos (*Macropus giganteus*) presenting with typical white coating of the lips gums, tongue and oesophagus. Due to the lesions and encrustations the young were reluctant to suckle. Failure to thrive on milk diets, prolonged episodes of diarrhoea and antibiotic therapy during hand-rearing were considered by Obendorf, (1980) to be significant predisposing factors to infection.

The characteristic lesions of primary ringworm have also been reported amongst marsupial species. *Tinea canis* and *Tinea mentagrophytes* often affect marsupials, and infection has been transmitted to human handlers (Stevenson and Hughes, 1988).

1.3.5 Protozoa

1.3.5.1 *Giardia* sp.

Giardia is an intestinal flagellate parasite of humans and various other animals. The species belongs to the Phylum Sarcomastigophora, Class Zoomastigophorea (Beaver *et al.*, 1984). This parasite perpetuates as environmentally resistant cysts which are responsible for the transmission of the parasite from host to host through ingestion, while the vegetative motile trophozoite which colonizes the small intestine but rarely the stomach, may be responsible for causing disease (Quincey *et al.*, 1992). Cysts may remain viable for long periods in the environment under favourable conditions (Feely *et al.*, 1990; Thompson *et al.*, 1993) while trophozoites are sensitive to environmental stresses such as osmotic shock, temperature changes, chemical disinfectants and pH (Adam, 1991; Lujan *et al.*, 1998).

In Australia, *Giardia duodenàlis* (synonymous with *G. lamblia* and *G. intestinalis*) (Thompson *et al.*, 1993), is the most commonly reported intestinal parasite of human populations (Meloni *et al.*, 1993). The reported prevalence of *G. duodenalis* varies widely depending on such factors as the method of detection, the population studied and geographic location (Horejs and Koudela, 1994). The prevalence of giardiasis in

some Australian Aboriginal communities can be as high as 82% in children (Swan and Thompson, 1986; Meloni *et al.*, 1993). In Tasmania, studies have been conducted on the prevalence and epidemiology of human parasitic infections by Goldsmid, 1980; Goldsmid 1981; Hawkesford and Goldsmid, 1995; Kettlewell *et al.*, 1998, which have also indicated the importance of human giardiasis in the state.

Manifestations of the disease vary from asymptomatic carriage to severe diarrhoea and malabsorption (Mills and Goldsmid, 1995). The development of clinical disease appears to be influenced by the immune system of an infected host (Ebert, 1999). Epidemiological, clinical, and experimental observations suggest that *Giardia* is capable of eliciting a host immune response (Belosevic *et al.*, 1983, Ungar and Nash, 1987; Heyworth, 1992), yet the relevant immunological mechanisms that combat the parasite infection are not completely understood (Farthing, 1995; Faubert, 1996; Stager, 1998). In humans, infections with *G. duodenalis* can be chronic among hypogammaglobulinaemic patients, suggesting a role for humoral immunity and, to a lesser extent, cellular components in the elimination of the parasite (Hughes *et al.*, 1971; Ament and Rubin, 1972; Granot *et al.*, 1998). Information derived from mouse models suggest immunity to *Giardia* seems to be especially linked to specific IgA which may act by binding to trophozoites so inhibiting their adherence to the intestinal mucosa (Den Hollander *et al.*, 1988; Ravdin *et al.*, 1988). Humans infected with *Giardia* produce specific circulating antibodies such as IgM against the parasite that may play a role in resistance to infection by mediating complement fixation (Snider, *et al.*, 1985; Nash and Aggarwal, 1986; Snider and Underdown, 1986; Belosevic *et al.*, 1994). Further, *Giardia*-specific serum IgG has been shown to be involved in antibody-dependent cellular cytotoxicity with polymorphonuclear leukocytes (Den Hollander *et al.*, 1988). Recent studies suggest antigenic variation of the surface antigens of *Giardia* might have a role in helping the parasite evade host immunological defences (Nash, 1992).

Transmission of giardiasis occurs predominantly via the faecal-oral route with as few as 10 to 100 cysts being required to initiate human infection (Rendtorff, 1954). Cysts are ingested with food or water, including chlorinated water, which has been contaminated by infected human or animal faeces (Thompson *et al.*, 1993). Once ingested, excystation begins in the acidic environment of the stomach passing into the proximal small intestine. Following exposure to pancreatic secretions, excystation is triggered which releases trophozoites (usually two per cyst) when the conditions for survival and proliferation are optimal (Farthing, 1996; Lujan *et al.*, 1998). The trophozoites lodge on the surface epithelium, embedded in mucus, deep in the small intestinal crypts where they divide by binary fission. At this point the trophozoites are motile and may be responsible for diarrhoea and malabsorption (Farthing, 1996; Lujan *et al.*, 1998). To complete the life cycle some of the trophozoites encyst within an extracellular cell wall, and following encystation, the rigid cysts are excreted with faeces. The stimulus for encystation remains controversial. There are several suggestions to explain what may promote encystation including high concentrations of bile salts (Gillin *et al.*, 1987; Campbell and Fauber, 1994), an atmosphere containing nitrogen and carbon dioxide or an environment that is poor in cholesterol (Lujan *et al.*, 1998).

Giardiasis has been reported world-wide in companion animals and was classified by WHO as a zoonotic disease in 1979 (Faubert, 1988). Numerous water and foodborne outbreaks have been described worldwide, although debate continues on the importance of pets and other mammals as possible reservoirs for human infection (Bemrick and Erlandsen, 1988; Davies *et al.*, 1993). Even with successful transmission experiments of zoonotic *G. duodenalis* to humans (Majewska, 1994), the epidemiological implications remain unresolved. Molecular research may help to clarify these implications. Isoenzyme analysis (Meloni *et al.*, 1988) and DNA fingerprinting (Nash *et al.*, 1985; Upcroft, 1990; Carnaby *et al.*, 1994; Carnaby *et al.*, 1995) has detected a marked genetic diversity within species of *G. duodenalis* (= *G.*

intestinalis). Interpretation of results from DNA analyses suggest that there is a close similarity between *G. duodenalis* (= *G. intestinalis*) obtained from humans and those of domestic and wild animals, further supporting the view that giardiasis is a zoonosis. Both Butcher *et al.*, (1994) and Carnaby *et al.*, (1995) provide evidence that more than one DNA fingerprint profile can be obtained in cloned isolates from the same human infection reinforcing the suggestion that mixed infections with genetically diverse *Giardia* isolates can occur in humans.

Since the acceptance of giardiasis as a zoonosis interest in the range of animal hosts harbouring *G. duodenalis* has increased (Thompson *et al.*, 1993). The first case of clinical giardiasis in the pet dog of a patient with *Giardia* in Tasmania was reported by Davies *et al.*, (1993), who suggested that an animal reservoir for human infection exists in Tasmania. Subsequent investigations have revealed the presence of *G. duodenalis* cysts in faecal samples of dogs, cats and marsupials, including possums, wallabies, wombats, Tasmanian Devils and bandicoots in Tasmania (Milstein, 1993; Davies 1995a; Milstein and Goldsmid, 1995). *G. duodenalis* has not yet been reported from prototherian mammals (monotremes). The clinical significance of *Giardia* in marsupials is not known but in a variety of local domestic animals it is known to cause diarrhoea, particularly in captive and farm animals, including alpacas (Kettlewell, 1995)

1.3.5.2 Haemogregarines

There has been much confusion in the literature of the taxonomic classification and nomenclature of haemogregarines(Levine, 1982). Haemogregarines belong to the protozoan Phylum Apicomplexa. Members of the family are not actually gregarines despite their name, but are assigned to the order Eucoccidiomorpha (Levine, 1982). There are four genera which belong to this family; *Haemogregarina, Karyolysis, Hepatozoon,* and *Cyrilia.* Differentiation of the first three genera is based on characteristics of their oocysts and not those related to their names. Members of the

genus *Haemogregarina* produce oocysts which are relatively small and contain 8 naked sporozoites. In contrast *Hepatozoon* spp., have large oocysts with many sporocysts that contatin 4-16 or more sporozoites each.

The original description of an *Hepatozoon* species in bandicoots was that of Welsh and Dalyell, (1909) which they took to be members of the Family Haemogregarinidae, describing them as *Haemogregarina peramelis*, Family Haemogregarinidae (Welsh and Dalyell, 1910). Wenyon, (1926) placed all the haemogregarines of mammals in the genus *Hepatozoon* and this species became known as *Hepatozoon perameles* (cited by Mackerras, 1959a). The reclassification among species found in marsupials was based on the identification of gametocytes infecting either erythrocytes or leucocytes. The species was further redescribed, and extracellular as well as intracellular gamonts were documented in the short-nosed bandicoot (*Isoodon obesulus*) from Innisfail in northern Queensland (Mackerras, 1959a). Mackerras, (1959a) found the infections were less common in animals from the southern Queensland areas of Gympie and Brisbane. No *Hepatozoon* species have to date been reported from the temperate regions of Australia.

The problem of differentiating *Hepatozoon* species from those within the genus *Haemogregarina* based solely on gametocyte morphology and morphometry of gamonts in the blood, has resulted in much taxonomic confusion (Desser, 1990). Furthermore, less is known of the life-cycles and modes of transmission of *Hepatozoon* species in temperate vertebrates than of related species of blood parasites in tropical and subtropical countries (Krampitz, 1981). Levine, (1982), attempted to bring some order to the Family Haemogregarinidae, introducing new names to replace homonyms or previously unnamed species. Unfortunately, the life cycles and vectors of the majority of species are unknown and assignment to any of these genera remains tentative.

The sporogonic stages in the definitive (invertebrate) host of other species, specifically the size of the oocysts and the presence or absence of the sporocysts, is the primary criterion for differentiating the two genera (Desser, 1990). There have been suggestions by Desser, (1993) that erythrocytic merogony may be a useful criterion for differentiating the genus *Hepatozoon* from *Haemogregarina* species (cited in Davies, 1995b). Although it is believed that arthropod ectoparasites act as vectors of the genus *Hepatozoon*, examinations of ixodid ticks, mites, and fleas from infected tropical bandicoots in Queensland and northern NSW failed to reveal life-cycle stages (Mackerras, 1959a). Knowledge of these aspects of haemogregarine biology is thus essential for the determination of the taxonomic status of this protozoan. The clinical significance of this parasite in marsupials is unknown, as is its potential role in contributing to the marsupial's susceptibility to other infections. Most adeleine haemogregarines are usually non-pathogenic although some species of the genus *Hepatozoon*, for example *Hepatozoon canis*, have been known to cause disease in their hosts (Olsen, 1974).

1.3.5.3 *TOXOPLASMA GONDII* AND TOXOPLASMOSIS

1.3.5.3.1 Taxonomy

Toxoplasma gondii is an obligate intracellular protozoan first discovered by Nicolle and Manceaux in 1908 (Faust *et al.*, 1970). The parasites were first isolated from the spleen and liver of a North African rodent, *Ctendactylus gundi*, hence the species name (Garcia and Bruckner, 1993; Lin *et al.*, 1990).

T. gondii belongs to the Phylum Apicomplexa, Class Sporozoa and Subclass Coccidia (Levine *et al.*, 1980; Beaver *et al*, 1984). The coccidia can be divided into two distinct groups - the enteric coccidia (e.g., *Eimeria*) which have a monoxenous (single host) lifecycle, and the cyst-forming coccidia (e.g., *Toxoplasma*), which have a heteroxenous life cycle (Cawthorn, 1989). There are presently seven genera of cyst-forming coccidia belonging to the Phylum Apicomplexa; these include

Toxoplasma, Besnoitia, Hammondia, Sarcocystis, Neospora, Frenkelia and *Cystoisospora* (Holmdahl, 1995). The anterior pole of the asexual stages of this parasite group has a characteristic apical complex, which resembles that of other members of the Phylum Apicomplexa (Schwartzman and Krug, 1989). The Class Sporozoa is conveniently divided into two subclasses, the Coccidiomorpha and Piroplasmia (Baker, 1969). The sub-class Coccidiomorpha includes a very important group of parasitic organisms including those which cause coccidiosis and malaria in humans.

Six species belonging to the genus *Toxoplasma* were identified by Beaver *et al.*, (1984) but only one of these, *T. gondii*, is known to infect humans. *T. hammondi* was found in cats and mice while the other species are confined to reptiles and amphibians. By the beginning of the 1960's, *Toxoplasma* had become known as an important cause of abortion in sheep, but the natural life cycle of this protozoan was still very unclear and the definitive host remained unknown. Transmission of *T. gondii* from the faeces of cats, leading to the acknowledgment of the cat family (Felidae) as the definitive host was finally described by Hutchinson, (1965). Although the complete life cycle only takes place through the Felidae, this parasite has a cosmopolitan distribution, with high prevalences in a large range of birds and mammals and all warm-blooded species may act as intermediate hosts (Miller *et al.*, 1972). Infection with *T. gondii* (toxo-infection) occurs more frequently than the clinical disease (toxo-disease) (Frenkel, 1973; Mills and Goldsmid, 1995); nevertheless *T. gondii* is recognised as an important pathogen of humans, domestic animals and wildlife.

1.3.5.3.2 Tachyzoites

The obligate intracellular tachyzoites are the invasive asexual forms of *T. gondii* and are prevalent during acute infection. The tachyzoites are eukaryotic cells, containing nucleus, golgi apparatus, ribosomes and mitochondria. Tachyzoites measure 7x3 μM

and are known to actively participate in the invasion of a host cell (Ferguson and Hutchison, 1987a). This process appears to be dependent on the extracellular ion concentration and motility of the tachyzoites. Interestingly, Werk (1985) found that tachyzoites can invade host cells at a rate of 15-40 per second, less than that required for phagocytosis. Tachyzoites may be found intra - or extracellularly, singly or in clumps. There is no well-defined parasitic membrane around clumps of tachyzoites within a cell (Garcia and Bruckner, 1993).

1.3.5.3.3 Bradyzoites

Bradyzoites predominate during the chronic, latent phase of infection. *In vivo* the forms encyst 8-10 days after ingestion (McLeod *et al.*, 1991). They differ from tachyzoites in their ability to withstand the presence of pepsin, hydrochloric acid and trypsin thus enabling their survival. They also have slower generation times than tachyzoites. Bradyzoites have stage-specific antigens but do not express the major tachyzoite membrane proteins p22 and p30 at detectable levels (Kasper and Ware, 1985; Omata *et al.*, 1989; Tomavo *et al.*, 1991). Cytoplasmic vacuoles contain the carbohydrate amylopectin, which provides energy during latency (McLeod *et al.*, 1991). Dubey and Frenkel, (1976) indicated bradyzoites were the only stages with the ability to initiate enteroepithelial cycles and transform into oocysts in the feline intestine. Little is known about the factors leading to the formation of bradyzoites or to oocyst formation and work on genes expressing selection or transition of bradyzoites is preliminary (Wong and Remington, 1993).

Both asexual (schizogony) and sexual reproduction (gametogony) of *T. gondii* occur in the intestine of the cat. The developmental cycle in the cat takes approximately 20-24 days after the ingestion of oocysts, but only three to five days after the ingestion of tissues containing cysts of the protozoan (Garcia and Bruckner, 1993). In the sexual life cycle, five forms of the organism, A-E, have been shown to exist prior to gamont formation. It is not known what feature of the feline intestine

permits oocyst formation, yet it is here that the cysts are disrupted, releasing the bradyzoites. The bradyzoites are slow proliferative stages and they invade the feline enterocytes, differentiating into male and female gametes in the asexual cycle known as gametogony (Dubey and Frenkel, 1972). The fusion of micro- and macrogametes results in zygote formation. Once a zygote has developed, a protective wall is laid around it to form the oocyst. Nearly all cats shed oocysts in faeces after ingestion of tissue cysts, whereas fewer than 50% of cats shed oocysts after ingesting tachyzoites or oocysts.

1.3.5.3.4 Oocysts

The ovoid unsporulated oocysts shed in cat faeces measure 9-11 µM wide by 11 to 14 µM long. The oocysts become infectious one to five days after being excreted into the environment where sporulation occurs. The infected cat may shed oocysts for as long as one to two weeks and as many as 10 million per day can be shed. Cats infected for the second or subsequent time rarely pass oocysts (Dubey *et al.*, 1970a; Wong and Remington, 1993). A sporulating oocyst contains two sporocysts containing four sporozoites, and cellular division produces eight infective sporozoites. The sporulated oocysts are remarkably resistant and are able to survive in soil for several months with extended viability when humid conditions prevail (Yilmaz and Hopkins, 1972; Wong and Remington, 1993). Oocysts can be successfully recovered from water or moist soil after many months (Kasper and Ware, 1985) and are resistant to many chemical agents, including disinfectants. A continual process of drying and heat will render them non-viable (Wong and Remington, 1993).

1.3.5.3.5 Life Cycle: Tissue Cysts and the Intermediate Host

The principal forms of transmission of toxoplasmosis is *via* tissue cysts (containing bradyzoites) or oocysts (containing sporozoites). Primary infections in humans result from ingestion of raw or undercooked meat and vegetables that contain cysts

as well as by direct contact with cat faeces (Wong and Remington, 1993) and water (Benenson *et al.*, 1982; Aramini *et al.*, 1999). Captive marsupials are equally susceptible to *T. gondii* by contact with contaminated environments or foodstuffs (Arundel *et al.*, 1979; Johnson *et al.*, 1988). Infection may also take place *in utero* or neonatally.

Following the ingestion of *T. gondii* cysts the outer walls are disrupted by enzymatic processes and infective stages of bradyzoites or sporozoites are liberated into the intestinal lumen (Wong and Remington, 1993). Once liberated, they rapidly invade and multiply within the surrounding cells of the lumen where they become tachyzoites, an actively dividing intracellular form (Wong and Remington, 1993). The tachyzoites appear to reside in cytoplasmic vacuoles, multiplying with a generation time of five to ten hours to form rosettes which eventually lead to cell rupture and liberation of the parasites (Wong and Remington, 1993). The cells proliferate by endodyogeny, a process where two daughter cells form from each tachyzoite (Jones and Hirsch, 1972, Jones *et al.*, 1972; Makioka *et al.*, 1993). Thereafter, the parasites invade contiguous cells and spread throughout the host via the blood and lymphatic systems.

In human and experimental infections it has been noted that the tachyzoites produce excreted-secreted antigens (ESA) that are highly immunogenic (Darcy, *et al.*, 1988; Decoster *et al.*, 1988; Charif *et al.*, 1990; Cesbron-Delauw and Capron, 1993). Both antibody-dependent and cell-mediated protective immune responses against experimental toxoplasmosis are induced by these antigens (Darcy *et al.*, 1988; Charif *et al.*, 1990). It was observed by Charif *et al.*, (1990) that the secreted antigens are first stored in tachyzoite-dense granules and are then released inside the parasitophorous vacuole following host cell invasion.

Tissue cyst formation within the intermediate host occurs in organs and tissues particularly within striated and heart muscle cells. Cysts characteristically appear in

the brain of chronically infected animals (Sharma and Dubey, 1981). *T. gondii* cyst formation within specific host cell types is consistent with observations made for other cyst-forming coccidia. Tissue cysts have been detected 6-10 days post infection in laboratory animals (Conley and Jenkins, 1981).

Based on interpretations of post-mortem findings, *T. gondii* cysts rupture intermittently and give rise to lesions (Frenkel and Escajadillo, 1987). It is unclear why numerous cysts disintegrate simultaneously. More importantly, each cyst may contain several thousand bradyzoites and can measure up to 50 μM in diameter and can range from 10-100 μM in length (Wong and Remington, 1993). Wong and Remington, (1993) observed that bradyzoites occasionally escape from cysts without completely disrupting the cyst wall. Tissue cysts contain stored glycogen and are surrounded by an elastic argyrophilic and periodic acid Schiff-positive wall (Dubey *et al.*, 1970b). The outer membrane of tissue cysts is acquired from the host cell and the parasite and is an adaptation of the lining of the original parasitophorous vacuole (Ferguson and Hutchison, 1987b). Numerous tubular structures are present beneath the cyst wall in older cysts. The cyst wall acts as a barrier to physical forces while still facilitating an exchange of nutrient material between host cell and the enclosed bradyzoites (Ferguson *et al.*, 1994). The wall allows the exchange of nutrients from the host cell to the multiplying bradyzoites and contributes to their maintenance throughout chronic infections.

Due to the complex host-parasite relationship it is unknown whether tissue cysts are solely intracellular or persist extracellularly as conflicting data have been published. It was strongly suggested by Ferguson and Hutchison, (1987a) that tissue cysts found in the brain were exclusively intracellular. Frenkel and Escajadillo, (1987) noted that intact tissue cysts were commonly found in brain sections with no apparent inflammatory response. Ferguson *et al.*, (1991) indicated that during the early stages of cyst rupture and development of microglial nodules there is a marked influx of

inflammatory cells at the site, indicating a prompt immune response to leakage of cyst contents.

For many animal species the exacerbation of latent toxoplasmosis becomes more frequent when immunity is depressed. Toxoplasmosis affecting the central nervous system (CNS) is one of the leading causes of death in AIDS patients (Levy *et al.*, 1985; Luft and Remington, 1992; Mills and Goldsmid, 1995). Toxoplasmic encephalitis (TE) can develop in patients undergoing immunosuppressive therapy (Ambroise-Thomas and Pelloux, 1993; Joynson, 1993), with characteristic manifestations including necrotizing encephalitis, myocarditis and pneumonitis (Chamberland and Current, 1991). It is believed that toxoplasmic encephalitis is caused by recrudescence of a latent infection and some authors have postulated that the brain itself is the original site of recrudescence (Frenkel, 1985; Hofflin and Remington, 1985; Frenkel and Escajadillo, 1987). The immune system thus plays a critically important role in the prevention of this disease as TE is more often found in immunocompromised patients.

1.3.5.3.6 Routes and Sources of Infection

Sporulated oocysts are the main source of infection with *T. gondii* for herbivores, carnivores and omnivores (Frenkel, 1973). Oocysts play a major role in the dissemination of infection due the large number excreted by the cat and the resistant nature of the sporulated oocysts. Small amounts of soil, such as that caught under the fingernails after digging is sufficient to contain an infective dose of *T. gondii* oocysts (Frenkel *et al.*, 1975). Outbreaks of toxoplasmosis of sheep in Britain showed a clear association between the spreading on pastures, of straw bedding, presumed contaminated by cats, with outbreaks of toxoplasmosis in sheep (Faull *et al.*, 1986). Infection as a result of direct contact with cats is considered particularly low as they keep themselves meticulously clean and freshly-shed oocysts are unsporulated and therefore non-infectious (Dubey, 1986).

Tissue cysts (containing bradyzoites) are the main source of infection for carnivores and omnivores. Most cats are naturally infected from their prey. Humans and captive carnivores are infected by eating raw or undercooked meats, including lamb/mutton, pig and goat (Dubey, 1994) (Figure 1.1). Horse meat and beef can infect humans, but are less likely to contain *T. gondii* tissue cysts than other meat sources (Al-Khalidi and Dubey, 1979; Dubey and Thulliez, 1993).

Oral ingestion of tachyzoites is not a reliable route of infection as they are less resistant to gastric acid than tissue cysts or oocysts (Jacobs *et al.*, 1960). However, tachyzoites may be found in a wide variety of secretions and body fluids (Saari and Räisänen, 1977).

Goat's milk has been shown to be a (rare) source of human infection with *T. gondii* (Dubey, 1980). Experiments with cats and dogs have indicated a possibility of infection when the animals were lactating their young (Chamberlain *et al.*, 1954; Dubey and Hoover, 1977;).

Transplacental infection occurs when pregnancy and primary infection of the mother coincide (Estaban-Redondo and Innes, 1997). For humans, sheep, goats and a lesser extent pigs, transplacental infection is important as a cause of abortion and neonatal mortality and morbidity (Dubey, 1986; Dubey, 1990a, 1990b; Charleston, 1994).

1.3.5.3.7 Transport hosts

Normally mammals and birds can act as intermediate hosts for *T. gondii* but invertebrates such as earthworms, flies, cockroaches, land snails and dung beetles may act as transport hosts, by carrying the parasite on the surface of the body or passing unchanged through the gut (Miller *et al*, 1972; Frenkel *et al.*, 1975; Saitoh and Itagaki, 1990). The possibility that *T. gondii* can be ingested and survive in blood-sucking arthropods, although unlikely, cannot be discounted (Woke *et al.*,

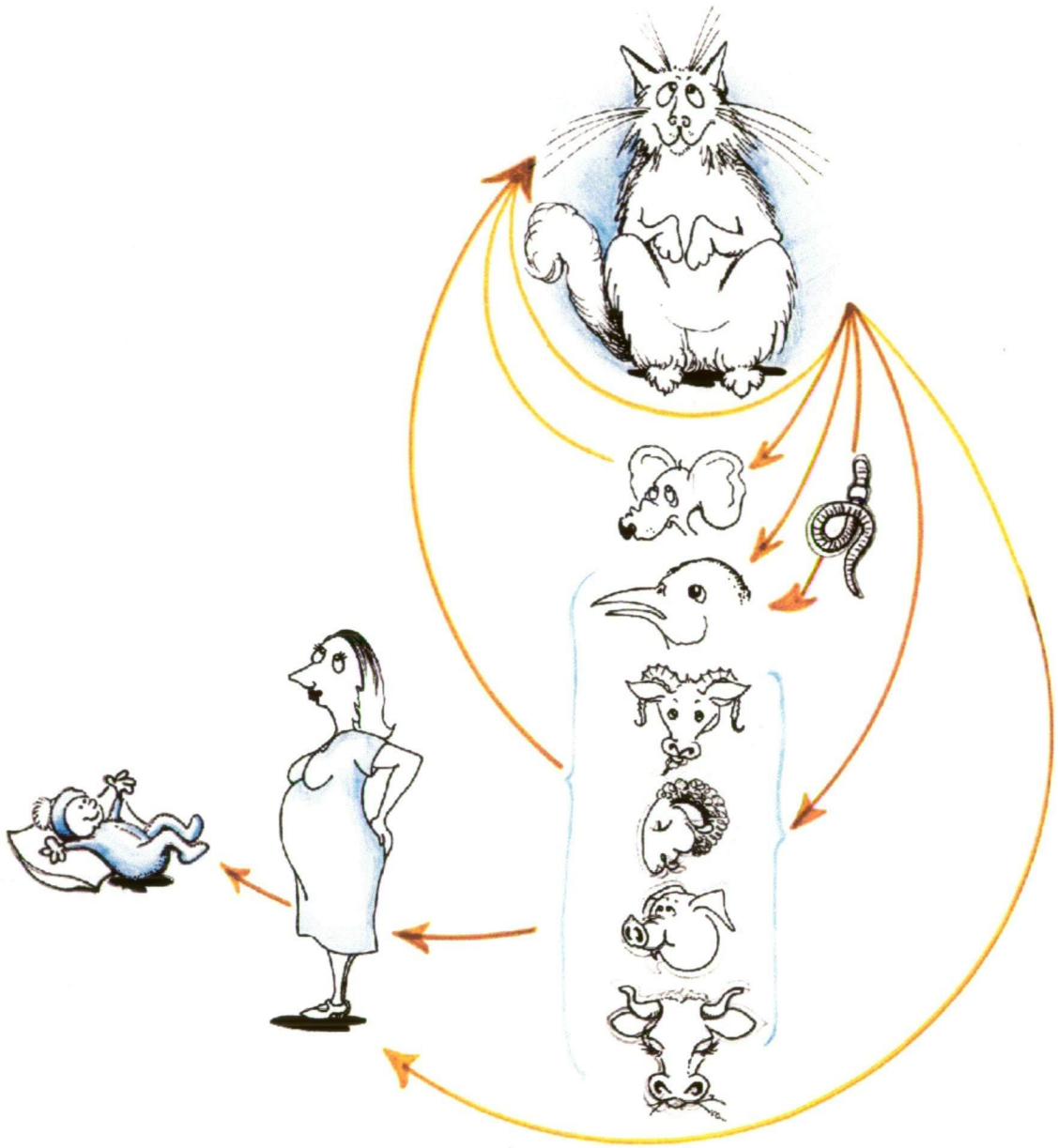


Figure 1.1. The epidemiology of toxoplasmosis. Transmission begins with the definitive host of *Toxoplasma gondii*, the domestic cat, with humans tangentially infected by the ingestion of oocysts or tissue cysts from intermediate host ingestion. Cats can become infected also by ingesting oocysts passed by other cats or ingestion of tissue cysts. Invertebrates such as earthworms may act as transport hosts.

1953; Frenkel, 1973). *T. gondii* may also be carried by reptiles but does not replicate in these or poikilothermic species (Miller *et al.*, 1972; Garcia and Bruckner, 1993). Mammals may also pass oocysts intact through their digestive system, to be deposited and then re-ingested by another individual (Dubey and Frenkel, 1973; Beverley *et al.*, 1975).

1.3.5.3.8 Disease caused by *T.* gondii

In normal individuals infection caused by *T. gondii* is often subclinical (Frenkel, 1973), sometimes termed "toxo-infection" (Mills and Goldsmid, 1995), with the individual seroconverting and in most cases harbouring the tissue cysts for years if not the lifetime of the host (Leighty, 1990). The common time for clinical disease (or toxo-disease), to occur is usually at the time of initial infection. Clinical presentation is often mild, with non-specific clinical signs of lethargy, mild pyrexia and lymphadenopathy (Beattie, 1957; Mills and Goldsmid, 1995). The more severe manifestations of toxoplasmosis involve respiratory signs, neurological signs and death. In some species toxoplasmosis can result in abortion or severe congenital defect in the neonate despite few or no clinical signs or symptoms in the mother (Estaban-Redondo and Innes, 1997). Recrudescence can occur due to immunosuppression of the host including that produced by stress, drugs or interaction with infection by other organisms (Seitz and Trammer, 1998). Local reactions may occur following rupture of tissue cysts.

Different species vary greatly in their response to a given strain of *T. gondii*. The "low virulence" S48 strain used in a vaccine for sheep, caused fatal systemic toxoplasmosis in naïve wallabies, even at half the dose administered for sheep (Lynch *et al.*, 1993). Early attempts by Reddacliff *et al.*, (1993), showed that only two out of five wallabies survived oral inoculation of *T. gondii* when earlier challenged with oocysts of the closely-related protozoan *Hammondia hammondi*.

Such variations within a species make the attempt to develop a vaccine a very difficult task.

1.3.5.3.9 Toxoplasmosis in *P.* gunnii and other Australian Marsupials

Toxoplasmosis has been documented in experimental rabbits in Australia since 1937 and in domestic animals since the 1950's (Wickham and Carne, 1950), but the pathogenicity of *T. gondii* infection in different species varies widely. The most vulnerable animal groups include the Australian marsupials and the New World primates, where acute infection is often fatal to the host (Cunningham *et al.*, 1992; Miller *et al.*, 1992; Innes, 1997).

Toxoplasma gondii is one of the most important protozoans to endangered marsupials, particularly vulnerable species such as *P. gunnii* (Obendorf and Munday, 1990). The only definitive host of this protozoan parasite is the introduced cat, *Felis catus*, which was introduced to the Australian continent with European settlement over 200 years ago or possibly even earlier by seafarers and shipwrecks (Wagner, 1997). This suggests that many of the Australian marsupials, like the New World primates, only encountered *T. gondii* with the advent of Europeans and their cats.

A report of the sudden death from toxoplasmosis of wallaroos (*Macropus robustus*) in a central Californian zoo colony, highlighted the growing problem of outbreaks in marsupials in captive and in the wild due to *T. gondii* (Boorman *et al.*, 1977). Since then, there have been numerous reports of toxoplasmosis in grey kangaroos (*Macropus giganteus*), red kangaroos (*Megalia rufus*), the Tammar wallaby (*Macropus cupenii*) and black-faced kangaroos (*Macropus fuliginosus melanops*) (Dubey *et al.*, 1988a). Other reports have included captive wombats, koalas and dasyurids (Dickens, 1975; Canfield *et al.*, 1990a, 1990b, 1990c; Hartley *et al.*, 1990).

Many species of small Australian wild animals have been reported with antibodies against *T. gondii* (Gibb *et al.*, 1966; Attwood *et al.*, 1975; Obendorf *et al.*, 1983; Johnson *et al.*, 1990). A greater prevalence of seropositive animals appears to be found in cooler, moister areas of Australia (Jakob-Hoff *et al.*, 1983). In the temperate island of Tasmania, Johnson *et al.*, (1988) detected a high prevalence of *T. gondii* antibodies amongst the Tasmanian pademelons (*Thylogale billardierii*) and Bennett's wallabies (*Macropus rufogriseus rufogriseus*). Toxoplasmosis has been recorded in both wild and captive macropods and may be seen sporadically (Kronberger and Schuppel, 1976) or in epizootics (Schroder and Ippen, 1976; Patton *et al.*, 1986). Clinical toxoplasmosis in macropods is usually fatal (Johnson *et al.*, 1989), but the existence of considerable numbers of live, healthy macropods seropositive for *T. gondii* (Dubey, *et al.*, 1988b; Johnson *et al.*, 1988) suggests that large outbreaks of disease associated with high mortality, may be a result of stress allowing the activation of latent infection. Unfortunately, toxoplasmosis has been described in wild bandicoots which often show no clinical signs or significant antibody levels before their sudden death (Pope *et al.*, 1957; Obendorf *et al.*, 1990).

Pope *et al.*, (1957) first described toxoplasmosis in a wild-trapped bandicoot *Isoodon obesulus* (= *I. thylacis)*, with splenomegaly as well as notable brain and lung lesions. In laboratory-kept bandicoots (*I. obesulus*), two infants dying of toxoplasmosis had severe oedematous pneumonia, with free *T. gondii* being noted in lung smears. One animal showed no signs or symptoms prior to death, while the other was seen ill and fitting (Cook and Pope, 1959). A study by Lenghaus *et al.*, (1990) in *P. gunnii* found potentially fatal generalised toxoplasmosis in more that 10% of animals at post-mortem examination (PME). The few *P. gunnii* reported with seropositive titres had IgG antibodies, suggesting that infections were chronic and latent (Miller *et al.*, 2000). In Tasmania, reports of naturally infected *P. gunnii* are often found with severe and debilitating neurological signs. At PME the animals are thin and reported to have severe pulmonary congestion (Obendorf, personal communication).

Histologically, non-suppurative myocarditis, pulmonary congestion and oedema and non-suppurative encephalitis, with associated *T. gondii* tissue cysts, are notable (Obendorf and Munday, 1990). There may also be a link between *Toxoplasma* infection and ocular pathology in this endangered species (Miller *et al.*, 1993).

1.3.5.4 Miscellaneous Protozoa

Klossiella species have been identified in numerous Australian marsupials (Barker, 1985). *Klossiella quimrensis* sp.n., were identified in the kidneys of both *P. gunnii* and *I. obesulus* during routine histopathological examination of tissues by Barker *et al.*, (1975). Apart from enlargement of the parasitised cell it appeared to cause no damage (Speare *et al.*, 1984).

Eimeria species have been detected in *P. gunnii*, with the original reports being from *P. gunnii* of Victoria. The intestinal coccidia were found to be common and appeared to be nonpathogenic (Obendorf and Munday, 1990). *Eimeria* spp., are usually involved in coccidial diarrhoea with fatal infections being recorded in wallabies and kangaroos in both wild and captive situations (Winter, 1959; Speare *et al.*, 1982; Barker *et al.*, 1989). A few species have been recorded from annelids, arthropods (mostly centipedes) and protochordates suggesting a possible source of infection for omnivorous animals (Baker, 1969).

Sarcocystis spp., are apicomplexan parasites that typically have a heteroxenous life cycle, alternating between herbivorous and omnivorous or carnivorous vertebrate hosts (Levine and Tadros, 1980; Levine 1986; Mills and Goldsmid, 1995). Secondary (intermediate) hosts are prey species such as sheep, cattle, pigs and mice whilst primary (definitive) hosts include cats, dogs, snakes, owls and humans (Langham and Charleston, 1990). An intermediate host can harbour the cysts in muscle tissue without significant reaction, whilst the more acute clinical condition of *Sarcocystis* infection is related to intravascular development often resulting in foetal

abortion and reproduction failure particularly in domestic stock (Langham and Charleston, 1990).

Munday *et al.*, (1978) screened a large range of Australian wildlife, reporting the presence of *Sarcocystis* or related organisms in most species, including *P. gunnii*. The high incidence of *Sarcocystis mucosa* amongst Tasmanian pademelons (*Thylogale billardierii*) and Bennetts wallabies (*Macropus rufogriseus*) suggested that dasyurid marsupials were possibly definitive hosts for *S. mucosa* in free-ranging Tasmanian macropodids (Jakes, 1998). This included one or more of the carnivorous dasyurids, *Dasyurus maculatus* (Tiger Cat), *Dasyurus viverrinus* (Eastern Quoll) and *Sarcophilus harrisii* (Tasmanian Devil) (Jakes, 1998). These marsupials and in particular *S. harrisii* may play an important role in perpetuating the transmission cycle of *Sarcocystis* spp., in Tasmania. It was also suggested by Jakes, (1997) that the alternative definitive hosts may include bandicoots and marsupial native cats, as although these animals usually prey on insects and small lizards, they are also opportunistic scavengers.

1.3.6 Helminth infection

Helminths are worm parasites that belong in the main, to three main invertebrate phyla, including Platyhelminthes, Nematoda and Acanthocephala (Mercer and Chappell, 2000). A variety of gastrointestinal nematodes have been described and listed from *P. gunnii* and during an investigation of *P. gunnii* by Obendorf and Munday, (1990) numerous endoparasites were recovered and identified (Appendix 11.2).

Nematodes belonging to the family Trichostrongylidae are commonly recorded in bandicoots. *Peramelistrongylus skedastos* was first described in *P. gunnii* by Mawson, (1960). Similarly *Labiobulura inglis* was described by Mawson, (1960) in *P. gunnii* and other bandicoots, including *I. obesulus* from Tasmania. During the

investigation by Mawson, (1960), all southern Australian bandicoots examined were found infected with a species of nematode belonging to the family Rhabditoidea and including *Parastrongyloides australis* similar to that found by Mackerras, (1959b) in bandicoots from Queensland. Other nematodes have been isolated from various tissue and organs of *P. gunnii* (Appendix 11.3). The acanthocephalan, *Australiformis semoni*, has also been recorded in *P. gunnii* (Spratt *et al.*, 1991).

The only cestode recorded to date from *P. gunnii* has been *Hymenolepis peramelidarum* Nybelin, 1917 (Spratt *et al.*, 1991) and overall trematodes appear to be uncommon parasites in Macropodoidea with only three species reported in free-ranging hosts. Within *P. gunnii* only one has been reported, *Mehlisia acuminata* S.J. Johnston, (1913) from the small intestine (Obendorf and Munday, 1990; Spratt *et al.*, 1991).

1.3.7 Arthropod Infections

Numerous arthropod ectoparasites including mites, ticks and fleas have been reported from *P. gunnii* (Obendorf and Munday, 1990) (Appendix 11.3). Four species of mites have been recorded in both Tasmanian and Victorian *P. gunnii.* These include *Haemolaelaps domrowi, Haemolaelaps marsupialis, Mesolaelaps antipodianus* and *Mesolseasp asutraliensis.* Two species from the family Ixodidae have been identified from Tasmania, *Ixodes tasmanii* and *Ixodes cornuatus. P. gunnii* carry ticks mainly inside the ears and harbour fleas throughout the body fur (Mallick *et al.*, 1997b). Of the order Siphonaptera, several species have been identified including *Pygiopsylla hoplia, Pygiopsylea zethi,* and *Stephanocircus dasyuri.* The degree of infestation of fleas is variable amongst *P. gunnii* populations but in southern Tasmania, fleas were more common than ticks (Mallick *et al.*, 1997b). Male *P. gunnii* were also more likely to be infested with both arthropods than female *P. gunnii* (Mallick *et al.*, 1997b). Mosquitoes have also been commonly reported near bandicoot nesting sites (Mallick, personal communication).

1.3.8 Ticks and Toxin Related Paralysis

The ixodid ticks are a problem for both domestic and wild animals (Gemmell *et al.*, 1991) and hardticks are known to transmit a wide variety of potentially zoonotic bacterial, viral, and protozoan diseases. In domestic animals, heavy ectoparasitic infestations may have a debilitating effect on the host and are known vectors of haemoprotozoan and rickettsial diseases (Pierce [illegible], 1956; Stewart *et al.*, 1982; Gemmell *et al.*, 1991). *Ixodes tasmanii* is the natural vector of *Theileria perameles*, a haemoprotozoan parasite in short-nosed bandicoots (Gemmell *et al.*, 1991) and which has been noted to produce anaemia in the marsupial (Mackerras, 1959a). Gemmell *et al.*, (1991) in their work on juvenile *I. macrourus*, related heavy tick infestations to high juvenile mortality rates in the wild and in captivity. Several disease syndromes have been described as being associated with tick infestation including, anaemia, malaise, paralysis, allergic manifestations, loss of condition, and other local and systemic disturbances (Gemmell *et al.*, 1991).

Infestation with Australian paralysis tick, *Ixodes holocyclus* is a common problem for domestic animals and occasionally humans on mainland Australia (Doube, 1975; Grattan-Smith *et al.*, 1997). The engorging female tick injects a neurotoxin which causes progressive motor paralysis, respiratory depression and death in animals which have no immunity to the toxin (Doube, 1975; Grattan-Smith *et al.*, 1997). Although tick paralysis has been observed in native fauna, these animals are mostly not usually affected. The natural hosts of *Ixodes holocyclus* include bandicoots, wallabies, kangaroos and other marsupials. *I. cornuatus*, which is found in Tasmania, is less dangerous than *I. holocyclus* but has been recorded as causing a mild form of paralysis in animals (McManus, personal communication).

1.4 THE MARSUPIALS AND THEIR IMMUNE SYSTEM

1.4.1 The Marsupial Immune System

The Peramelidae have a unique accelerated life history and chorioallantoic placenta which from an evolutionary perspective raises the questions with respect to their development and onset of immune competence in comparison with other marsupials. Yet studies of the marsupial immune system have been limited to a small number of species with few studies focussing on bandicoots. In many instances the studies on bandicoots have been limited because of the paucity of species available in large numbers for laboratory experimentation. Some marsupial species are now established as self-sustaining colonies, thus providing a good supply of laboratory animals of known source and genetic constitution. Animal models more commonly used, include the Australian brush-tail possum (*Trichosurus vulpeula*) (Moriarty and Thomas, (1983), the quokka (*Setonix brachyurus*) (Yadav and Eadie, 1973), the Virginia opossum (*Didelphis virginiana*) (Rowlands and Dudley, 1968), and the South American gray short-tailed opossum (*Monodelphis domestica*) (Fadem *et al.*, 1982; Hubbard *et al.*, 1991). Recently, work by Wilkinson *et al.*, (1992; 1994) following threats to the survival of koalas (*Phascolarctos cinereus*) from increasing disease, successfully established baseline immunological parameters, while Bourne, (1997) attempted to investigate the cell-mediated immune responses of Bennett's wallabies (*Macropus rufogriseus rufogriseus*).

1.4.1.1 Lymphoid Organs

The thymus plays a pivotal role in the development of immunocompetence and the marsupial thymic development in pouch young is relatively rapid, suggesting a rapid development of cell-mediated immunity (Cisternas and Armati, 1999). Fraser, (1915) first observed the foetal thymus of *P. gunnii* and confirmed the observations of Johnstone, (1898) on adult material, that this peramelid possessed a bilobed thoracic thymus but no cervical thymus (cited by Tedman, 1990). Yadav, (1973) reported this feature in both *P. gunnii* and *Isoodon obesulus* (Southern Brown

Bandicoot) and also noted that the absence of a cervical thymus was a feature of all polyprotodont marsupials. However, herbivorous marsupials (diprotodonts) including *Trichosurus vulpecula* and *S. brachyurus* have two distinct glands, the thoracic and cervical thymus. These animals characteristically have a small number of pouch young contained within a large, deep pouch, with a rich and varied bacterial profile. The cervical thymus of the developing young in this group is much larger than its thoracic counterpart at all stages, and its differentiation occurs more rapidly. To meet the immunological threat in the pouch, investigators consider a gland in the neck would enlarge more easily than one which was confined to the thorax (Turner, 1994).

In the Northern Brown Bandicoot (*Isoodon macrourus*) the rapid thymic maturation reflects its shorter pouch life, therefore, reflecting its need for some degree of immunocompetence at an earlier stage (Cisternas and Armati, 1999). At birth its thymus gland is small and lacks a clear-cut cortex, medulla and is devoid of Hassall's corpuscles. In the mature marsupial, these organs histologically show comparative morphological and functional characteristics of the immuno-lymphatic organs of eutherians (Yadav, 1973; Marchalonis and Schluter, 1994). In marsupials however, differentiation and maturation of all major organs occurs during pouch life, while in eutherians, development takes place *in utero* (Mossmann, 1972 cited by Cisternas and Armati, 1999).

Thymectomy and its effect on lymphoid tissue of the quokka, *S. brachyurus* was investigated by Ashman *et al.*, (1975a). It was noted that cervical thymectomy retarded development of lymphoid tissue and was strongly associated with the development of protective immunity in pouch young of this species (Stanley *et al.* 1972; Jurd, 1994). Yadav *et al.*, (1972a) noted that early (pre-day 10) cervical thymectomy, or later (pre-day 20) thoracic and cervical thymectomy depleted circulating lymphocyte counts and there appeared to be slow recovery after complete

thymectomy. A similar lymphocytic depletion was seen when thymectomy was performed on *Didelphis marsupialis*, and this marsupial possesses only a thoracic thymus, similar to the bandicoot (Jurd, 1994). In other investigations thymectomy has also resulted in lymphocytic depletion in lymph nodes and spleen, a reduced antigen response, a transient abolition in response to sheep red blood cells (SRBCs), a reduced response to mitogen phytohaemagglutinin (PHA) and a shortened lifespan (Ashman *et al.*, 1975b; Jurd, 1994).

1.4.1.2 Mitogen Responsiveness

Measurement of proliferative responses of lymphocytes is a fundamental technique widely used to assess biological responses to various stimuli. Mitogenic responses using different concentrations of mitogens such as phytohaemagglutinins (PHA), which primarily stimulate T-cell proliferation, are one of the most commonly used parameters for immune system evaluation. Lymphoid cell isolation and the proliferative responses of these cells to mitogens have been analysed in several marsupials. Ashman *et al.*, (1976) studied the Tammar wallaby (*Macropus eugenii*) and mitogen responses throughout the pouch life of *S. brachyurus*. The pouch young of *S. brachyurus* at 60 days, gave a good peripheral blood and lymphoid tissue response to T-cell mitogens phytohaemagglutinin (PHA) and concanavalin A (Con A), whereas the pouch young at 100 days gave a good response with the T- and B-lymphocyte mitogen, pokeweed (PWM). The evidence supports the claim by Jurd, (1994) that the presence of cell-mediated T-cell immunity is critical in protecting the pouch young.

1.4.1.3 Major Histocompatibility Complex (MHC)

Very little is known about the marsupial major histocompatibility complex (MHC) which in eutherians regulates a number of cellular immune responses. Infante *et al.*, (1991) found in marsupials that T- and B-peripheral blood cells were only weakly

stimulated by allogeneic and xenogeneic (mouse) lymph node cells in mixed lymphocyte reactions (MLR's), despite the prompt rejection of tail skin allografts and xenografts. This suggested substantial polymorphism at the Class I MHC gene but poor Class II gene polymorphism existed in these animals. Jurd, (1994) speculated that a lack of positive selection mechanisms for MHC Class II polymorphism may correlate with a lack of a "true" chorio-allantoic placenta. Very few in-depth studies have been undertaken in this area, though Stone *et al.*, (1987) successfully used the *Monodelphis domestica* model to detect seven Class I antigens which were controlled by genes in at least two autosomal loci. Hybridization of four DNA fragments from this marsupial with mouse Class I MHC cDNA fragments (pH2IIa) revealed 70% similarity of sequence (Jurd, 1994).

1.4.1.4 Cell-Mediated Immunity

Cell-mediated immunity has been examined in the marsupial *D. virginiana* by skin reactivity to dinitrochlorobenzene (DNCB) and to tuberculin, agents known to cause Type IV delayed (cell-mediated) hypersensitivity reactions in placental animals (Jurd, 1994). The investigation also included migration inhibition factor (MIF) production. Relevant cell-mediated immune responses occurred later and were less intense than those measured in other laboratory animals such as guinea-pigs, and the transfer of immunity by cells was less efficient. Laplante *et al.*, (1969) transplanted skin allografts in *D. virginiana* which were quickly rejected, whilst Infante *et al.*, (1991), noted similar rejection of allografts and xenografts in the opossum (*Monodelphis domestica*).

1.4.1.5 Humoral Immunity

Jurd, (1994) noted that there is little standardization of immunisation regimes or antigens in the study of the humoral immune responses in marsupials. Initial studies of the primary humoral immune responses have utilized sheep red blood cells and

haemolytic titration, *Salmonella* Adelaide flagella and bacteriophage ØX174 with good primary responses to the antigens particularly for *M. domestica* (Yadav, 1973; Croix *et al.*, 1989). These responses are variable between marsupial species with secondary responses varying with species and antigen. The primary response to SRBCs by *M. domestica* was recorded to be long-lasting but the secondary response in comparison with placentals appeared weaker and more transient (Croix *et al.*, 1989; Jurd, 1994).

1.4.1.6 Immunoglobulins

Studies of some metatherian marsupials suggest the structure, function and production of immunoglobulins (Ig) closely parallel those seen in the eutherian mammal (Rowlands and Dudley, 1968; Bell, 1977; Blandon, 1987). Bell, (1977) reported the distribution within Australian and American marsupials of heavy and light chain antigenic markers identified by antisera to purified *S. brachyurus* immunoglobulins. Markers for IgM and IgG_2 constant region determinants as well as light chains appear to be widely distributed in Australian and American species. Despite the fact that the marsupials from Australia and America are two geographically separated groups that have evolved in isolation during the last 130 million years, the results of Bell, (1977) indicate there is a long-term structural conservation of some immunoglobulins within these marsupials.

Detailed analysis of the distribution of marsupial IgG_2 by Bell, (1974a; 1974b) suggest that there has been a gradual acquisition of markers with time. Its presence in species separated for so long indicates that IgG_2 was possibly the ancestral IgG, present before the divergence of these separate lines. IgG_1 is more obscure and limited to a small group of closely related diprotodont marsupials suggesting more recent origins. The range of isotypes recorded in marsupials includes, IgM, IgG_1, IgG_2, IgA and IgE. Most reports on the molecular weights or nature of these

immunoglobulins indicate that they appear similar to those of placentals, but the investigations are limited and no direct comparisons can be made. Comparative analysis of amino acid sequences for IgY, IgA, IgE and IgG from various animal species reveal that *M. domestica* IgE, IgG and IgA on the phylogenetic tree, form branches clearly separated from their eutherian counterparts (Aveskogh and Hellman, 1998). However, they still conform to the general structure found in eutherian IgE, IgG and IgA. More recent data at a molecular level has compared the evolution of IgG between orders of marsupials confirming the appearance of IgG prior to the metatherian/eutherian divergence (Lucero *et al.*, 1998; Belov *et al.*, 1999). Belov *et al.*, (1999) isolated a 1518 nucleotide cDNA clone when screening the Australian brushtail possum *T. vulpecula* mesenteric lymph node cDNA library with the South American *M. domestica*, immunoglobulin gamma heavy chain constant region (C gamma) probe. The sequence from the Australian marsupial was 70% identical at the amino acid level with the American marsupial sequence, but less similar to the eutherian mammals (45-50%).

1.4.1.7 Cytokines

Very little information is available on marsupial cytokines due to the slow progress of research in this area (Harrison and Wedlock, 2000). Due to vast improvements in protocols and reagents, some progress has been made in the past decade. Initial steps were taken by the extraction of interleukin-1 (IL-1) from the South American opossum, *Monodelphis domestica,* obtained from lipopolysaccharide (LPS) - stimulated macrophages and keratinocytes and had a molecular weight of 15-17 kDa (Brozek *et al.*, 1991). Brozek *et al.*, (1991) showed that the product was unable to stimulate mouse thymocytes and the cytokine was not antigenically similar to murine IL-1. More recently, in 1996, the first Australian marsupial cytokine, brushtail possum tumour necrosis factor (TNF), was cloned and sequenced (Wedlock *et al.*, 1996). The genomic nucleotide sequence and chromosomal position of the interleukin 5 (IL5) gene has been described for the model marsupial *Macropus*

eugenii (Tammar Wallaby) (Hawken *et al.*, 1999). A number of other marsupial cytokine genes have been isolated and found to be structurally and functionally similar to their eutherian counterparts (Harrison and Wedlock, 2000). As this research begins to progress, much remains to be learnt about the structure, function and evolution of marsupial cytokines.

1.4.1.8 The Pouch Young and Maternal Antibody

Marsupial young are born at a very early stage of development and continue their growth as neonates attached to a teat usually enclosed in a pouch, or marsupium. The young are born antigenically inexperienced and immunologically incompetent, which is in contrast to eutherians which are born immunologically competent but antigenically inexperienced (Jurd, 1994).

There is little information regarding transplacental transfer of passive humoral immunity in marsupials. There is also no clear evidence of antibody transmission across the marsupial yolk-sac placenta. It appears that once the vulnerable neonate reaches the pouch, maternal antibodies are transferred *via* milk and a rapid development of immunocompetence protects the immature offspring.

To determine how immunity is transferred, early studies of female marsupials such as *Monodelphis domestica* and the Australian *Setonix brachyurus*, were experimentally hyperimmunized with sheep red blood cells (SRBCs) before and during gestation (Stanley *et al.*, 1966). In *S. brachyurus* detectable antibody to SRBCs could not be elicited until 10 days of age (Stanley *et al.*, 1966). Similarly, antibodies were present only in the newborn *M. domestica* when allowed to suckle from their mothers (Samples *et al.*, 1986). This suggested that the neonate in the pouch, was enclosed in what has often been described as a "second womb" (Jurd, 1994). Like eutherians, the transfer of antibodies in the milk is principally confined to the IgG class, although Bell *et al.*, (1974b) examined the immunoglobulins of

marsupial milk and other secretions and described an IgA-like molecule prominent in these fluids. IgE was also considered to be transferred in this way (Bell *et al.*, 1974b). An investigation of three species of kangaroos, *Macropus robustus*, *M. rufus*, and *M. giganteus* detected very low IgG serum levels during the first 90-100 days of pouch life, and it was assumed that pouch young were largely protected by milk-transferred antibodies during this period (Deane and Cooper, 1984). After this period the offsprings' own antibodies took over the defensive role. Turner, 1994, considered that the marsupials, like their eutherian counterparts, vary in their capacity for pre- and postnatal transfer of immunoglobulin to their young.

Marsupial offspring must survive the potential pathogens contained in the pouch environment. In some species the gut of the pouch young is first colonized at about 10 days of age, when immunocompetence first becomes apparent. To overcome this problem, pouch secretions contain immunoglobulin-like substances which reduce the pouch flora of pregnant females thus giving the pouch young an opportunity to adapt to their environment and become immune-competent (Yadav *et al.*, 1972b). Maternal protection of the neonatal marsupial appears to occur primarily through immunoglobulins secreted into milk, and probably for the duration of development of lymphoid tissue (Old and Deane, 2000). The overall understanding of the development of immunocompetence and maternal protection of young marsupial is far from complete (Old and Deane, 2000).

1.5 SUMMARY

In view of the merits of marsupials as models for reproductive, developmental and immunological studies it is often considered to be disappointing that current research has failed to keep pace with eutherian work (Turner, 1994). The fact that large proportions of marsupials are highly susceptible to acute diseases such as toxoplasmosis suggests current investigations of the immunobiology of these infections are necessary. Many marsupial species are prone to diseases and the

decline in marsupial populations caused by their susceptibility to infection is an important factor that significantly influences the ecology of the species (McKenzie, 1981). A better understanding of the capability of marsupials, such as *Perameles gunnii,* to respond to pathogens is important for both diagnosis and treatment of diseases in marsupials. In addition, such information would provide the basis for monitoring success of new management practices and perhaps a future use of vaccination.

The objectives of this thesis sought firstly, to assess the infections of *P. gunnii*, their prevalence and clinical significance. Secondly, to study the immune system of *P. gunnii* in relation to its ability to overcome infections and finally, to evaluate the potential role of *P. gunnii* as a reservoir for human infections.

Chapter 2. General Materials and Methods (methods used in two or more chapters)

2.1 *P.GUNNII* ANIMALS

Due to ethical issues involved in working with vulnerable species such as *P. gunnii* certain limitations were placed on this project by the animal Ethics Committee of the University of Tasmania and Tasmania's Parks and Wildlife Service, Department of Primary Industries, Water and Environment (DPIWE). Due to the small pockets of *P. gunnii* existing across southern Tasmania, restrictions were placed on access to various sites and animal populations. In many instances permission was only given for a capture and release investigation. This was restricted to capture and handling the animals for the shortest period of time, but long enough to take a blood sample, collect faecal specimens, collect and count ectoparasites and obtain details of overall health. Permission for a limited number of animals to be held in captivity, was only given to those captured on a private golf course bordering on a reserve in Redwood Village, Kingston in southern Tasmania. The one exception was for experimental work in Chapter 3, when permission was given for the capture of six animals from the Huon Valley.

All animals were used with the approval of the Animal Ethics Committee of the University of Tasmania. Trapping and handling of animals was in accordance with established guidelines and protocols of Tasmania's Parks and Wildlife Service permit to take protected wildlife under National Parks and Wildlife Act 1970 and the Crown Lands Act, 1976. Individual permit numbers were sought for each investigation. Animals held in captivity were housed in the Central Animal House at the University of Tasmania. Animals were housed either in individual housing pens or sterile cages. Each animal had free access to water and a special diet was provided. Variations to this diet were made according to the requirements of the investigation.

2.1.1 Description of Trapping Sites

Capture and release investigations were conducted on private land in the Huon Valley of south-east Tasmania, Australia. Two permanent trapping grids were placed in the Huon Valley region at Grove and Huonville (Grids I: 43°03'S, 147° 02'E; and Grid II 42° 59'S, 147° 05'E) in March 1992 by Tasmania's Parks and Wildlife Service. The grids were located along the Huon Highway and were separated by a distance of approximately 15 km. Each grid covered an area of approximately 0.20 km^2, including 11 trap-lines separated by 100 m, and each trap-line with 5 trap-stations separated by 50 m, giving a total of 55 trap-stations per grid (Mallick, *et al*, 1998).

The purpose of this joint study was for Tasmania's Parks and Wildlife Service to investigate the demography of two populations of *P. gunnii* in south-east Tasmania using live-trapping on the two permanent grids, to determine the prevalence of *T. gondii* and for the current investigation to investigate diseases in *P. gunnii.* In addition, a comparative study of road-kills on adjacent segments of highway and soil diggings as a population index for *P. gunnii* was made (Mallick *et al.*, 1998, 1997b).

The Huon Valley is an agricultural district and a well established apple-growing area of southern Tasmania. It includes a mosaic of small townships, with pastures and patches of remnant eucalypt woodland and forest. The topography at each site was distinctly different, with Grid I being situated on the lower hill-slopes bordering the Huon Valley (Figures 2.1, 2.2) while Grid II was situated on the gently undulating river-flats on the valley floor (Figures 2.3, 2.4). Both areas included open pasture (approximately 70% of total area), while the remainder was of native open-woodland and forest. Grid I included small areas of open sedgy-woodland and dense wet sclerophyll forest while Grid II had a single patch of open grassy-woodland. The surrounding country was also distinctly different, with Grid I being situated within a matrix of large, contiguous patches of dense bushland broken by open areas made up

Figure 2.3. Grid II, Grove. An open grassy-woodland with a native and introduced plant understorey.

Figure 2.4. Grid II, Grove. An open matrix of continuous pasture-land broken by small isolated patches of remnant bush.

Figure 2.1. Grid I, Huonville. Typical open pastures with patches of eucalypt woodland and forest.

Figure 2.2. Grid I, Huonville. Open sedgy-woodland and dense wet sclerophyll forest.

of individual paddocks. In contrast, Grid II occurred within a more open matrix of continuous pasture-land broken by small isolated patches of remnant bush along its hilly terrain.

During the three year study, parts of both grids were utilized by the owners at various times for grazing sheep and cattle. A single house was built on the edge of each site toward the end of the study period. Native and introduced vegetation was removed at various intervals, particularly gorse (*Ulex europaeus*) and blackberries (*Rubus fruticosus*). At one end of Grid I, a third had to be abandoned in June 1994, due to interference of traps by dogs. The area was replaced with a new section of an equal distance from its other end-boundary, and included similar habitat to the area abandoned.

The trapping site at the Redwood Village, Kingston (Tasmania, Australia: 43°S, 147°03'E) golf club bordered on the edge of a reserve. Bushland was mainly dry sclerophyll dominated by eucalypts and a dense understorey (Figure 2.5). The main components of vegetation species were *Eucalyptus amygdalina* (Black Peppermint), *Leptospermum scoparium* (Manuka), *Acacia stricta, Epacris impressa* (Heath), *Leucopogon virgatus, Banksia marginata, Lomandra longifolia, Aotus ericoides* (Golden Pea) and *Dillwynia sericea.* European species bordered the fairway offering shade and lush greenery (Figure 2.6). The bandicoots lived in the bushland but ventured out onto the greens or the fairway to dig for earthworms and feed on insects.

2.1.2 Trapping Grids at Huon Valley

Trapping in the Huon Valley consisted of five-night trapping sessions conducted four times a year in March, June, September and December, from March 1993 until March 1996. Mascot wire cage traps (Mascot Wire Works, Enfield, New South Wales, Australia) (200 x 200 x 560 cm) were used and baited with peanut-butter sandwiches (Figure 2.7). To maximise capture success, trap stations were located

Figure 2.5. Trapping site at Redwood Village, Kingston. Dry sclerophyll bushland dominated by eucalypts and a dense understorey.

Figure 2.6. Redwood Village, Kingston. Native and European plant species border the fairway offering shade and protection to animals.

Figure 2.7. A secure Mascot trap.

Figure 2.8. To maximise capture success, trap stations were located within or adjacent to patches of ground-cover.

within or adjacent to patches of ground-cover wherever possible (Figure 2.8). All traps were partly sealed at one end with durable plastic to provide animals with protection from the elements and secured at the site with wood or rocks to avoid being moved or rolled. Traps were set during the morning of the first day of a trapping session, left open overnight, then cleared and re-set the following morning. Traps were removed after the fifth night of trapping. No problems were encountered during the trapping period and all individuals were extremely quiet whilst in the traps. When approaching the traps, the animals were not greatly disturbed or upset. They appeared to have suffered no physical injury on account of the traps, while other species like the Potoroo, (*Potorous tridactylus*), tended to suffer a large amount of physical damage as they tried to escape.

Seven native mammal species were captured on the grids including the Eastern Barred Bandicoot, *Perameles gunnii.* These included the Southern Brown Bandicoot (*Isoodon obesulus*), Long-nosed Potoroo (*Potorous tridactylus),* Eastern and Spotted-tail Quolls (*Dasyurus viverrinus* and *D. maculatus*), Swamp Rat (*Rattus lutreolus*), Common Brushtailed Possum (*Trichosurus vulpecula*), and Tasmanian Bettong (*Bettongia gaimardi*). In addition, two introduced species, black rats (*Rattus rattus*) and domestic and feral cats (*Felis catus*) were caught at both Grids, as was one Tiger snake!

All *P. gunnii* when first captured were ear-marked with a tattoo number, unique to Tasmania's Parks and Wildlife Service records (Figure 2.9). Animals were weighed and sexed. Routine linear measurements were made using Vernier calipers on individuals using similar standards employed by Wood-Jones (1924) and Lyne and Verhagen (1957) (cited by Moloney, 1982), for obtaining estimates (Figure 2.10). Records were made of each animals head length (back of skull to tip of nose), left ear (base of pinnae to tip), foot length (back of tarsus to tip of claw), tarsus length (back of tarsus to joint with meta-tarsi) and testes width and testes length for the males.

Figure 2.9. On first capture *P. gunnii* were ear-marked with a tattoo number for future identification.

Figure 2.10. Routine linear measurements were made using Vernier calipers on *P. gunnii.*

Pouch-young, the number, sex, head length of young, nipple size and location were recorded for the female. The general condition of the animals was noted including the state of the pelage, injuries and ectoparasites. The number of ticks on the ears was recorded and the degree of infestation by fleas was estimated as 'low', 'moderate' or 'high'. Similar procedures were performed on autopsy cases with a post-mortem report completed with special reference to *Toxoplasma gondii* (2.1.3).

2.1.3 PARKS AND WILDLIFE SERVICE POST-MORTEM REPORT FOR *P. GUNNII* WITH SPECIAL REFERENCE TO *TOXOPLASMA GONDII*

Species.....

Body Weight.....

Routine measurements.....

Age: (if possilbe at least adult, sub-adult and juvenile).....

Sex:.....

Please collect 2 to 4 mL of heart blood

Histology to be taken when possible:
Heart - Kidney -
Lung - Pancreas -
Liver - Tongue -
Adrenal - Spleen -
Brain -
Skeletal muscle (thigh, loin and shoulder) –

Test requested: Histology – selected tissues
Serology – Toxoplasma DAT/MAT

- Notify District Officer if any other interesting findings as gross examination and hold carcass.

2.1.4 DIETARY NOTES FOR YOUNG AND CAPTIVE BANDICOOTS

(Courtesy: Diane Green, Parks and Wildlife Services, Hobart, Tasmania)

Formula

1 scoop of Digestelact to 60 mL boiled water

2 drops of Infant Pentative

2 Drops of Vitamin E

1/8 of a teaspoon of Glucodin (1/4 tsp of Glucodin for first 24 hours after arrival)

Vitamin E used was Bioglan – Micelle E

Solids

Lean raw beef finely chopped or minced

Kitten Whiskettes (softened with water but not mushy)

Well-cooked lamb, chicken or beef

Apple, currants, sultanas, cheese (mild cheddar), peanuts (unsalted), raw carrot, sweet potato, cooked potato, hard boiled egg (yolk and white), sweet grapes, bread and peanut butter, rolled oats, goat pellets and wheat. (The two latter were seen eaten by wild bandicoots). These ingredients form the basic daily meal. 1/3 raw meat, 2/3 other ingredients. The other items on the list can be added to the basic meal for variety and the individuals preferences. Sultanas are a favourite and make a useful bribe. All ingredients are cut into small pieces and lightly sprinkled with "clean" soil. Soil must not contain manures or fertilisers and must be obtained from a cat-free area. On this diet alone their faeces resemble those of a natural fed wild bandicoot.

Insects *These insects appear to be their favourites.

Huntsman spiders, corbi grubs, bush cockroaches, crickets, grasshoppers*, soldier beetles*, lacewings*, butterflies, moths* (all sizes and varieties), European wasps (dead or stunned), pupas (all types), Ichneumon, cockchafer beetles and larvae*, bees

(dead), click beetles, auger beetles, ladybirds, worms, Eucalypt leaf eating beetle* (*Paropsis* species), earwigs, pointed leaf bug, slaters, non "furry" caterpillars, any grubs found in rotting logs and common brown beetles* (names unknown). There are other types of insects and beetles that they will eat but names are unknown. They will not eat; Longhorn beetles, weevils, slugs, snails, any insect that gives off a strong acrid smell when squashed or threatened, even when no other insects are available. A large amount (30-35) Eucalypt leaf eating beetles can be eaten in one sitting with no adverse effects. The beetles have quite a high eucalypt content and smell, neither of which deters the bandicoots. They will eat flies, but preferable not to be given, as they often require worming when fly eating is encouraged.

2.2 GENERAL METHODS: BLOOD COLLECTION, PREPARATION AND STAINING

2.2.1 Blood Collection of *P. gunnii*: Samples (0.5 – 2 mL).

Blood collection was limited to young adults and females with no pouch young. Once captured the animal was restrained usually in a hessian bag or dark-cotton pillowcase and blood samples were immediately collected from the peripheral ear vein. The perimeter of the ear was sterilized with alcohol and the vessels dilated by light massaging. A marginal blood vessel was lanced with a blood lancet (Microlance: Becton & Dickenson) and the blood scooped into collecting vials Microtainer Brand with ethylene diaminetetraacetic acid (EDTA) anticoagulant (Becton Dickinson Vacutainer Systems, Becton Dickinson & Co., New Jersey, USA). Tubes were inverted several times to ensure thorough mixing of blood and EDTA and thereafter inverted at intervals not exceeding 5 minutes to minimise the risk of blood clotting. For serum collection, 0.5 mL of blood was collected into Microtainer serum separating tubes with a gel interface (Microtainer: Becton Dickinson & Co., Rutherford, New Jersey, USA). Bleeding was stopped by applying pressure to the site or by sealing the wound with petroleum jelly. Blood samples were processed within an hour of collection.

2.2.2 Large Volumes of Blood (>5 mL)

When larger volumes of blood were required, free-ranging animals following capture were taken immediately to a private veterinary clinic at Kingston where approximately 6 mL of venous blood was collected by venipuncture. Animals were kept one - two hours for observation and then released back to their environment several hours later.

Animals in captivity were bled with the help of staff at the Central Animal House at the University of Tasmania. Bandicoots were either immobilised by gentle restraint or evaluated as stressed animals and anaesthetised using mask induction and gas inhalation with isoflurothane. This was administered at 0.5 mL/min by a Komesaroff Analgesic Machine. Once the individual animal was anaethetised the concentration was reduced. Six millilitres of venous blood was collected by two techniques.

The first technique used was drawing blood by cardiac puncture. This procedure provided good samples of blood but was not appropriate for long-term experimental work, due to the long recovery period of the animals. To perform the technique special expertise and experience was required. The technique was considered too invasive for bandicoots and posed a potential health risk for animals where repeat samples were required.

The second technique used involved drawing blood from the jugular vein. The neck region and upper thorax was clipped and the skin cleaned with dilute povidone iodine scrub and alcohol swab. Blood was collected using a 22 G needle (Terumo, Terumo medical corporation, MD, USA) and 10 mL syringe. Blood was then transferred to preservative free EDTA (Vacuette, Greiner Labortechnik, Germany). After collection the tubes were inverted several times to ensure thorough mixing of blood and anticoagulant and thereafter inverted at intervals not exceeding 5 minutes, to minimise the risk of blood clotting. The blood samples were processed within an hour of collection. This technique was the best alternative for large blood samples from this small marsupial resulting in minimum injury or stress. Recovery was immediate and allowed for repeat sampling from the same animal.

2.2.3 Making and Staining Blood Smears with Leishman's and Leishman's Wright Mixtures

Blood was taken from mature adult *P. gunnii* only. Peripheral blood films were made directly from withdrawn blood prior to transference to a tube with EDTA anticoagulant. The slide (wedge) technique as outlined in Beck, (1992) and Mitruka and Rawnsley, (1977) was utilized in the laboratory and in the field. One drop of peripheral blood was placed on the end of a clean glass slide, then drawn along with the spreader slide at an angle of about 45° and left to dry (a stain of proper thickness dries quickly at room temperature and appears yellow).

During field work it was, in some instances, difficult to make a film on site or for the smear to dry, especially during adverse weather conditions such as snow or rain. In these instances the films were made from the blood in anticoagulant as soon as possible after collection. Canfield, (1998) advised this should be no longer than four hours after collection to avoid natural deterioration of blood cells and diminishing cell morphology.

2.2.4 Leishman's / Wright's: Modified Leishman's Stain

Wrights	100 mL	(5 g / 2.5 L)
Leishmans	50 mL	(7.5 g / 2.5 L)
43/45 stronger Wrights		(6 g /1 L)

- Leave for 2 months before using.

Leishmans	100 mg
anhydrous methanol	200 mL

- Mix Leishmans and methanol at 36°C for 30 minutes.

1) Stir overnight at room temperature or > 6 hours.
2) Strain and pour into BROWN bottle. Stain should be stored away from sunlight and brewed for 2 months before use.

2.2.5 Conventional Staining of Thin Blood Slides with Leishman's Stain or Leishman's-Wright Stain (Mitruka and Rawnsley, 1977)

When applying the Leishman's-Wright stain the preferred choice was the conventional manner as described by Mitruka and Rawnsley, (1977) rather than the dip technique. Cell counts and scanning of slides for details were performed x100 using oil immersion.

- Prepare Buffered water pH 6.8

1) Air dry blood slide and heat fix slide
2) Stain with Leishman's stain for 4-8 minutes
3) Add buffered water (pH 6.8) approximately until 3/4 buffer: 1/4 stain. Pour buffered water on slide and blow. Leave for 8 minutes.
4) Wash gently in water for 2 minutes, until the smear appears pink and translucent. The slide was kept horizontal and the scum floated off with distilled water to prevent the precipitation from sticking to the slide.
5) Leave slide to air dry before examination under oil immersion.

2.2.6 Giemsa Stain

1) Fix with methanol
2) Stain for 20 minutes with Giemsa diluted (1:20) with buffered water with pH 7.0-7.2.
3) Wash in water
4) Leave slide to air dry before examination under oil immersion.

2.2.7 Acridine Orange Stain (Cranston and Goldsmid, 1972)

This technique involves staining thick and/or thin blood films with the fluorochrome Acridine Orange-O and examining under ultraviolet light (BG-12 exciter filter).

Thin blood films

1)	Fix in methanol	3 s
2)	Stain in Acridine Orange made up to 0.01% in Sorensens Phosphate Buffer pH 5.4	3 s
3)	Wash in buffer	3 s
4)	Wash in buffer	3 s
5)	Rinse in distilled water	2 s
6)	Dry and examine under oil	

Thick blood film

•	Stain in Acridine Orange made up to 0.01% in Sorensens Phosphate Buffer (pH 5.4)	30 s
2)	Wash in buffer	3 s
3)	Wash in buffer	3 s
4)	Rinse in distilled water	2 s
5)	Dry and examine under oil	

2.2.8 Differential Leukocyte Count

For differential leukocyte counts a blood smear was prepared on a slide and stained as described in 2.2.3. The stained smear was then examined under the microscope. Counts were performed with movement of the slide towards the tail and the process finished when the minimum 100 leukocytes was differentiated. The differential percentages of neutrophils (N), lymphocytes (L), monocytes (M), eosinophils (E) and basophils (B) were calculated and used to determine absolute cell counts using automated systems.

2.2.9 Total White Cell Counts Using a Haemocytometer.

Leukocytes were enumerated by using an improved Neubauer counting chamber. To perform the total leukocyte count, blood was drawn to the 0.5 mark on a white cell diluting pipette, then Turk diluting fluid was drawn to mark 11, the dilution being 1:20. A drop was placed on one side of the double chamber. Three minutes was allowed for cells to settle before being counted in five squares. Calculations were performed as described in Beck, (1992).

2.2.10 Haematological Data Analysis using Automated Systems

Chilled EDTA blood was analysed by a Coulter counter Model S plus Phase II or Technicon H2 Automated Counter (Dublin, Ireland) within 2 hours of collection. Blood samples were not analysed if considered unsuitable ie clotted or collected in the field in excess of the recommended time.

White blood cells (WBC), red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), haemoglobin distribution width (HDW) and platelet count (PLT) were recorded, mean platelet volume (MPV), platelet distribution width (PDW), and platelet crypt (PCT).

2.2.11 Measurements of Cell/Parasite Dimension Using the Image Analyser

Measurements and image analysis of the parasite were conducted using a Sony monochrome CCD camera attached to an Olympus BH2 microscope. Images were recorded via the camera and captured by a Macintosh Power PC 7600/120 AV computer using the public domain NIH Image program (written by Wayne Rasband at the US National Institute of Health and available by anonymous FTP from zippy.nimh.nih.gov). A micrometer scale bar was calibrated to the number of pixels per micrometer. The dimensions of length, breadth and area were measured and tabulated.

2.3 ELECTRON MICROSCOPY

2.3.1 Alsever's Solution

Glucose	20.5 g
Trisodium citrate, dihydrate	8.0 g
Sodium Chloride	4.2 g
Distilled Water	to 1 L

Adjust the pH to 6.1 with citric acid before autoclaving at 126°C for 30 minutes. Solution may be stored frozen. Use four to one dilution of blood to solution.

2.3.2 Glutaraldehyde Fixatives

Fixatives are prepared using the following formula to achieve the final concentrations.

0.2 M cacodylate buffer	50 mL	50 mL
25% Glutaraldehyde in H2O (mL)	10 mL	16 mL
Distilled Water to make (mL)	100 mL	100 mL
Final concentration of glutaraldehyde	2.5%	4.0%

NB. Osmolarity of final solution is 0.1 M. This may be adjusted with sucrose, glucose or sodium chloride if necessary.

- Check pH before use as prolonged storage may reduce pH.

2.3.3 Cacodylate Buffer

1) Prepare a solution of 0.4 M concentration by dissolving 21.4 g sodium cacodylate [$Na(CH_3)_2AsO_2.3H_2O$] in 250 mL distilled water.

2) Prepare the 0.2 M cacodylate buffer using the following formulae:

0.4 M Sodium cacodylate	50 mL
0.2 M HCl	8 mL
Distilled water to make	100 mL

- pH of the buffer is adjusted with HCl.

2.3.4 Processing Cell Suspensions for Transmission Electron Microscopy

1)	Wash cells in buffer	5 min (x 2)
2)	Fix in 1% Osmium tetroxide (OsO_4) in buffer	30 min
3)	Wash with distilled water	5 min (x 2)
4)	Fix in 4% uranyl acetate (U/A) in water	30 min
5)	Dehydrate in ascending alcohol concentrations of 50, 70, 95 and 100%	5 min each
6)	Propylene oxide	5 min (x 2)
7)	50:50 Propylene oxide/Epon mix	18 h
8)	Change to 100% Epon Mix	4 h
9)	Change to freshly made up Epon Mix	4 h

10) Embed cell plugs in mould and place in oven at 60°C for 48 hours.

2.3.5 Preparation of Epon Resin Mix

Procure 812	11.66 g
DDS	3.30 g
NMA	8.41 g

- Combine reagents into a glass beaker under a fumehood and cover with paraffin upon transferring to stirrer.
- Use magnetic stirrer to mix for 15 min.

Add DMP 30	0.3 – 0.4 g

- Mix again with magnetic stirrer for 15 min until rich yellow

2.3.6 Staining of Grids for Transmission Electron Microscopy

1) Bring reagents to room temperature before staining.
2) Place wax support in watch glass with sodium hydroxide pellets and cover.
3) Emit one drop of 4% uranyl acetate (U/A) onto paraffin wax paper.
4) Place grid membrane side down onto the U/A for 30 minutes and cover.
5) Wash in distilled H_2O.
6) Transfer grid to one drop of lead acetate and cover for 4 minutes.
7) Immediately wash in distilled H_2O.
8) Dry by placing forceps on watch glass upon hotplate.

2.3.7 Scanning Electron Microscopy (Lê, 1996)

Blood was collected from animals into 1.5 mL collecting tubes filled to 0.75 mL with Alsever's solution to preserve the sample and prevent coagulation. A 50:50 ratio of blood: Alsever's solution was obtained before the sample was mixed gently by inversion. Vaseline was spread on the surface of the ear to assist in collecting droplets of blood. Polylysine coated coverslips served as a template to prepare the red blood cells. The coverslips were washed with distilled water and dehydrated with acetone before being critically point dried using CO_2 as the transmission fluid. The variation of the technique was to fix the blood smears with Osmium tetroxide vapour (Probing and Structure, Thuringa, Queensland), air dried and sputter coated

with gold. This was repeated after sonication. The coverslips were observed under the SEM (JSM-849, JEOL) at the Antarctic Division, Kingston. Prints for observation and comparison were made from ASA/18DIN negatives using a polaroid.

2.4 HISTOLOGY and IMMUNHISTOCHEMISTRY

2.4.1 Histopathological Procedures

Selected organs and tissues from animals that had died were fixed in 10% buffered formol-saline; histological specimens (prepared in the Discipline of Pathology, University of Tasmania) were embedded in paraffin, sectioned at 4 μM and stained with haematoxylin and eosin (H&E) for microscopic examination. Duplicate sections were prepared on Chrome Alum slides for immunohistochemistry.

2.4.1.1 Haematoxylin and Eosin Stain

1)	De-wax in xylene (x2)	2 min.
2)	Rehydrate in alcohols (100%, 95% and 70%)	2 min.
3)	Wash in running tap water (RTW)	2 min
4)	Haematoxylin	5 min
5)	RTW	2 min
6)	Blue in NH_3H_2O	20 s
7)	RTW	1 min
8)	Eosin / phloxine	3 min
9)	RTW	10 s
10)	Dehydrate in alcohols (70%, 95%, 100%, 100%)	10 dips in each
11)	Clear in xylene	20 dips
12)	Leave in xylene until time to coversip	

- Mount immediately after removal form xylene

2.4.2 IMMUNOHISTOCHEMICAL TEST FOR *TOXOPLASMA GONDII* IN TISSUES USING VECTASTAIN ABC RABBIT IgG KIT.

2.4.2.1 Materials required

- Chrome Alum slides
- Vectastain ABC Rabbit IgG kit (Vector Laboratories, Bulingame, California, USA).
- Sigma Fast DAB (Sigma Chemical Company, St Louis, USA).
- Rabbit-anti *T. gondii* serum was used as the secondary antibody (kindly supplied by Department of Primary Industry and Water Environment (DPIWE), Prospect, Tasmania).

2.4.2.2 Chrome Alum Slides

1) Place slides in racks and soak overnight in detergent

2) Wash in running water for several hours or multiple changes of water

3) 3 x 30 washes in MilliQ water

4) While wet, dip slides in chrome alum–gelatin solution for approximately 5 s.

Gelatin	2.5 g
Chromic potassium sulphate	0.25 g
MilliQ water	500 mL

- Heat water to dissolve gelatin and filter solution before use.
- Place the racks on paper towel, cover with the same, and leave to dry overnight.
- Store slides in slide boxes.

2.4.2.3 Gill's Haematoxylin Solution (Gill, 1974).

Haematoxylin	2 g
Sodium iodate	0.2 g
Aluminium sulphate	17.6 g
Distilled water	750 mL
Ethylene glycol	750 mL
Glacial acetic acid	20 mL

- Mix the distilled water and ethylene glycol and add the haematoxylin. Next add the sodium iodate followed by the aluminium sulphate and mix. Finally add the acetic acid and stir for 1 h at room temperature using a magnetic stirrer. The solution is ready for immediate use and should be filtered when required.

2.4.2.4 Scott's Blueing Solution

$MgSO_4$	10 g
$NaHCO_3$	1.75 g

- Dissolve the salts separately, mix, and make up to 500 mL with distilled water. Add 1 crystal of thymol.

2.4.2.5 Procedure for Immunohistochemical Test (Manufacture's Instructions) (Vector Laboratories, Bulingame, California, USA).

1) Cut 4-8 μM paraffin sections.
2) Deparaffinize in 100% xylene.
3) Hydrate tissues to PBS

100% ETOH	10 min
5% ETOH	5 min
70% ETOH	5 min
50% ETOH	5 min

- PBS (PBS = FA-PBS plus 0.1% crystal Bovine Serum Albumin: BDH, Poole, England).

4) Quench endogenous peroxidase in tissues with 3% hydrogen peroxide in 100% methanol for 30 minutes (10 mL of 30% hydrogen peroxide 90 mL of methanol).
5) Wash in PBS two times for 5 minutes each time.
6) Block non-specific binding with normal goat serum.
   (3 drops / 10 mLs PBS use Yellow bottle in kit for 20 minutes).
   Blot off excess serum (but don't let slide dry out).
7) Incubate for 30 min to 1 hour in Rabbit-anti *Toxoplasma gondii* serum (1:500 in PBS 100 μL in 50 mL PBS).
8) Wash in PBS two times for 5 minutes each time.
9) Add biotinylated antibody (1 drop / 10 mL PBS, use BLUE bottle in kit). Incubate for 30 minutes.

NB. Make up Vectastain reagent at this point. It must stand for 30 min before being used in the test (2 drops A/10 mL of 0.1 M carbondate buffer pH 9,5 then 2 drops B) use ORANGE bottle in kit, mix immediately.

10) Wash in PBS two times for 5 min each time.

11) Incubate for 30 min in Vectastain reagent made up as above.

12) Wash in PBS two times for 5 min each time.

13) Incubate in DAB reagent for 4 min.

14) 0.105 g (5 tablets) DAB/50 mL of 0.1M Tris pH 7.2

15) 0.02% hydrogen peroxide for 30% = 35 µL in 50 mL distilled water.

16) Mix equal volumes of A and B to get DAB reagent.

17) Wash in distilled water for 5 min.

18) Counter stain in Gill II haematoxylin for 45 secs.

- Blue nuclei in Scott's tap water substitute for 30 secs dilution 1:9. (10 mL solution / 90 mL water).

19) Dehydrate tissues

50% ETOH	2 min
70% ETOH	2 min
95% ETOH	2 min (x 2)
Xylene	2 min (x2)

20) Coverslip with Permount.

Total time approximately 4 hours and 30 minutes.

2.5 TOXOPLASMOSIS DIRECT (DAT) AND MODIFIED (MAT) AGGLUTINATION TESTS (Courtesy DPIWE, Tasmania)

This agglutination test used whole *Toxoplasma gondii* parasites to detect IgG and IgM antibodies to *T. gondii* in serum. Collected *P. gunnii* serum was treated with 2 mercaptoethanol, which destroyed IgM immunoglobulin (MAT test), then tested in parallel with untreated serum to detect total *Toxoplasma* immunoglobulin (DAT test). The difference in titres between the DAT and MAT identifies the amount of IgM antibody present. The test is used for any species when fluorescent conjugates are not available for IFAT test. Due to non-specific IgM in some sera, the interpretation of results must be done with caution.

2.5 1 *Toxoplasma* AD Antigen

- Antigene Toxo-AD and microtiter plate reagents (bioMerieux SA, Marcy-l'Etoile, France).
- Store Refrigerated. Use undiluted.

2.5 2 (1.4%) 2 Mercaptoethanol

2 Mercaptoethanol	1.4 mL
Phosphate buffered saline (pH 7.2)	to 100 mL

- Store for up to 4 weeks refrigerated in a brown glass bottle.
- 2 Mercaptoethanol should be used in a fume cupboard. Avoid contact with skin and breathing vapour.

2.5 3 BABS Buffer (pH 9.0)

NaCl	0.702 g
H_3BO_3	0.309 g
NaOH (1 Normal)	24 mL
Bovine serum albumin	0.4 g
Sodium azide	0.1 g
Distilled water	to 100 mL

- Store refrigerated

2.5.4 Procedure for Toxoplasmosis Direct (DAT) and Modified (MAT) Agglutination Tests (Courtesy DPIWE, Tasmania)

1) Add 25 µL of serum to each of 2 microfuge tubes.
2) Label both tubes with the sample accession number and identify one as the 2ME treated sample tube.
3) Add 0.175 mL of PBS to one tube and 0.175 mL of 1.4% 2 Mercaptoethanol to the tube labelled 2ME (this process must be performed in a fume cabinet).
4) Incubate tubes for 60 min at 37°C in a water bath.
5) Add 0.2 mL of BABS buffer to each tube (to give a serum dilution of 1/16).
6) Set up 2 rows of 5 micronic tubes for each sample.
7) Add 0.3 mL of BABS buffer to each tube except the first of each row.
8) Add the contents of the microfuge tube containing the serum diluted in PBS to the first micronic tube of row 1.
9) Add the contents of the microfuge tube containing the serum diluted in 2 ME to the first micronic tube of row 2.
10) Transfer 100 µL from mirconic tube 1 in row 1 into the second micronic tube in row 1, mix and transfer 100 µL from tube 2 into tube 3. Repeat from 3 to 4 to 5. These dilutions are for the DAT test.
11) Repeat step 10 for row 2. These dilutions are for the MAT test. Each row now has dilutions of 1/16, 1/64, 1/256, 1/1024, and 1/4096.
12) Transfer 25 µL of each dilution to one well of a microtitre plate (e.g. the two 1/16 dilutions in wells A1 and B1, 1/64 dilutions in A2 and B2 etc.)
13) Record on a work sheet (SEROF-003) all test details and sample accession numbers and positions of dilutions on the plate and note which row is the DAT test and which is the MAT test.
14) Vortex antigen vigorously, immediately before use, and add 25 µL of antigen to all test wells.

- Cover plate and leave at room temperature on a vibration free bench for 4-6 h.
- Read results using a magnified mirror microtitre plate reader.

Positive reaction = a mat of agglutinated *Toxoplasma* parasites covering more than half of the well base.

Negative reaction = sedimentation of the parasites in the form of a small button in the bottom of the well, or partial agglutination covering less than half of well base.

- Positive control of known titre is available from bioMerieux (Toxotrol A cat. # 75431).

2.6 EARTHWORMS AND SOIL CONDITIONS (Chapters 3 and 5)

Earthworms used were commercially reared Red earthworms *Lumbricus rubellus* and Blue earthworms *Perionyn excavatus* (All State Worm, P/L, Adelaide, South Australia). These were selected on their palatability for captive *P. gunnii* (personal communications, Melbourne Zoological Gardens). Earthworms were purged over a 2-3 day period prior to experimental work. Over 1000 earthworms were maintained during the study.

Appropriate soil conditions were prepared to stock the worms. The soil mixture comprised a seedling-raising mix with no fertilizers (Amgrow, Castlereigh, Tasmania, Australia) combined in a ratio of 1:1 with a pretreated peat and dolomite mix (30kg each) (Amgrow, Castlereigh, Tasmania, Australia). This pretreatment involved soaking 15 L of compressed peat moss combined with 500 g of dolomite in a hessian bag for 48 hours in gentle running water. Once drained the mixture was combined with the soil and sterilized by autoclaving at 115°C for 1 hour - repeated after 18 hours. The mix was allowed to cool and then divided into four purging boxes. Approximately 3 kg of soil mix was placed into four black plastic boxes which form part of a worm composting kit (Reln Worm Factory, Reln Plastic, Sydney, NSW).

2.7 PARASITE DETECTION - Faecal sample collections

All faecal samples from the field were collected into wide-mouthed, water tight, sterile containers with tight-fitting lids. Specimens brought back to the laboratory were promptly examined using the techniques below and fixed in 10% formal buffered saline. Specimens that could not be examined were refrigerated at 4°C until required for use.

2.7.1 Parasite Detection Using Direct Wet Mount

The direct wet mount is the most useful for the detection of motile trophozoites and larvae, as well as the protozoan cysts and helminth eggs. It allows the detection of parasites that do not concentrate well, and detection of blood or mucus. A small drop of 0.85% saline and a drop of iodine were placed on a glass slide. With an applicator stick a small portion of the faecal specimen was mixed in each diluent and a cover slip added. The mount was scanned at high dry (40x) objective. The iodine highlights the morphology of protozoan cysts showing nuclear detail and glycogen masses.

2.7.2 Wheatley Trichrome Stain (Smith and Bartlett, 1991).

Fresh faecal samples from native animals were smeared onto a slide, fixed and stained using the Wheatley trichrome staining method described by Smith and Bartlett, (1991). Microscopic examination was performed at high dry (40x) and oil (100x) objectives. Permanent stains such as trichrome are often used for the detection and identification of protozoan trophozoites and cysts, in particular *Giardia* (Smith and Bartlett, 1991). The stain most widely used is the Wheatley trichrome stain that is easy to perform and the reagents have a relatively long shelf life. *Giardia* cysts are more readily identified by their size (10-12 μM), and morphology (oval with a longitudinal axostyle). The disadvantage of this method occurs when there are low numbers of organisms in the specimen making it difficult to locate even with a prolonged search.

2.7.2.1 Stock Solution of Saturated Mercuric Chloride with Alcohol

Mercuric chloride	47.5 g
Distilled water	675 mL

- Heat to dissolve. Stopper tightly and stand at room temperature overnight and filter. Leave for several days before using.

2.7.2.2 Schaudinn's Fixative

Saturated solution Mercuric Chloride	40 mL
95% Alcohol	20 mL
Glacial Acetic Acid	3 mL

2.7.2.3 Trichome Stain

Chromotrope 2R	0.6 g
Light Green SF	0.15 g
Fast Green FCF	0.15 g
Phosphotungstic acid	0.7 g

- Mix all dry ingredients together

Acetic Acid (glacial)	1.0 mL

- Add to dry ingredients and allow to "ripen for 15-30 minutes

Distilled water	100 mL

- Add distilled water slowly at first while continually mixing to dissolve all stain.
- Store stain in a tightly sealed bottle at room temperature.

2.7.2.4 Acid Alcohol

90% Alcohol	10 mL
Acetic Acid	1 drop

2.7.2.5 Procedure for Wheatley Trichrome Stain

1) Smear fresh faecal sample onto a clean microscope slide
2) Place directly into Schaudinn's fixative and fix for 1-24 hours.
3) Place in 70% alcohol + iodine for 1-2 min to remove $HgCl_2$
4) Place in 70% alcohol for 1 min to remove iodine.
5) Place in 70% alcohol (procedure can be stopped at this stage).
6) Stain in trichome for 6 min (range 2-8).
7) Dip slide 2-3 times in acid alcohol.
8) Rinse twice in 95% alcohol to remove acid
9) Dehydrate for 1 minute in absolute alcohol.
10) Dehydrate in xylene for 10 min.

- Mount in DPX.

2.7.3 Formalin-Ethylacetate Sedimentation Concentration (Smith and Bartlett, 1991).

Formal ethyl acetate concentration is a modified sedimentation technique based on a solvent extraction technique described by Smith and Bartlett, (1991). This method removes large particles by a sieve and fats are collected in the organic phase (ethyl acetate), leaving a layer of sediment that can be examined microscopically for cysts as well as oocysts and eggs and concentrated in the aqueous phase (10% formalin).

Faecal samples were mixed with 10% formalin buffered saline and processed using a Johns Parafilter System, a commercially available filtering and centrifuging system. Concentration was followed by microscopic examination of a wet preparation of the concentrate mixed with a drop of iodine. The sample was viewed using high dry (40x) objectives.

2.7.3.1 Procedure of Formalin-Ethylacetate Sedimentation Concentration

1) Mix fresh or formalin preserved stool specimen thoroughly in a flat bottomed tube and fill to 10 mL mark with 10% phosphate buffered formalin. When using a fresh stool, allow to stand 30 minutes for fixation to take place.
2) Add 2-3 drops of Triton-X 100
3) Screw on lid tightly and shake tube vigorously for at least 15 s. For fresh samples, leave to fix in formalin for about 30 min.
4) Remove lid, attach blue filter and conical tube to flat bottomed tube.
5) Invert contents into conical tube through filter.
6) Remove strainer and flat bottomed tube and discard appropriately.
7) Add 3 mL, or up to 10 mL mark, of ethyl-acetate.
8) Replace lid and mix well for 30 s.
9) Centrifuge at 800 x *g* for 2 min.
10) Upon removing tubes from centrifuge, specimen should be layered.
11) Rim debris layer with an applicator stick and pipette off fat/ethyl-acetate layer into waste bottle and pour off supernatant, leaving the sediment.

- Place some concentrated specimen on microscope slide and mix with a drop of iodine and saline.
- Cover with coverslip and examine.

2.7.4 Zinc Sulphate ($ZnSO_4$) Flotation Method (Smith and Bartlett, 1991).

This method was used on formalin fixed concentrated faecal specimens to yield a clean preparation with the removal of debris. A specific gravity of 1:20 for the zinc sulphate solution is recommended in order to raise the protozoan cysts and helminth eggs to the surface of the meniscus. A slide was carefully placed across the surface of the meniscus and left for half an hour for eggs or cysts to move to the surface. The slide was carefully removed and a cover slip placed over the slide. The slide was viewed at high dry (40x) objective.

The zinc sulphate concentration method was originally described by Faust *et al.*, (1938) and continues to be performed on unfixed or formalin-fixed specimens particularly for the concentration of *Giardia* cysts (Smith and Bartlett, 1991). This procedure is considered the final diagnostic technique used for stool specimen examinations. The zinc sulphate solution distorts and degrades the cysts and eggs, so further diagnostic methods on a specimen cannot be performed to achieve good results. All staining and testing of specimens was performed before this technique was utilized.

2.7.4.1 Preparation of Zinc-Sulphate Solution

$ZnSO_4.7H_20$	386 g
Hot distilled water	400 mL

- Dissolve zinc sulphate in hot distilled water and leave to cool to room temperature.

Cold distilled water	400 mL

2) Place cold distilled water in a measuring cylinder and add zinc sulphate solution to cylinder. Check the specific gravity with a hydrometer or by calculation. Add 10 mL of water for each 0.002 the specific gravity must be reduced, mix thoroughly, and check the specific gravity.

For example	Specific gravity	= density $ZnS0_4$sol/density H_2O
	whereby density $ZnS0_4$	= mass/volume
	e.g. Density ($ZnS0_4$)	= 12.0 g/10 mL = 1.2 g/mL

$\therefore$ specific gravity ($ZnS0_4$)= (1.2 g/mL) / (1.0 g/mL) = 1.2

2.7.4.2 Technique 1

1) Mix sediment of faecal specimen processed using formal-ethyl acetate concentration technique with 5 mL zinc sulphate solution.
2) Fill conical tube with zinc sulphate solution until solution forms a meniscus just above lip.

- Place coverslip over miniscus and leave for 5-10 min.
- Place coverslip on microscope slide with a drop of iodine and saline and examine

2.7.4.3 Technique 2

1) Mix sediment of faecal specimen processed using formal-ethyl acetate concentration technique with 5 mL zinc sulphate solution.
2) Fill conical tube with zinc sulphate to 1 inch from rim.
3) Centrifuge 1300 x *g* for 1 min.
4) Place tube carefully in rack and fill to top with zinc sulphate solution to form a meniscus over rim.
5) Place coverslip over meniscus.
6) Leave for 10 min.
7) Place coverslip on microscope slide with a drop of iodine and saline and examine

2.8 PERIPHERAL BLOOD MONONUCLEAR CELL (PBMC) SEPARATION FROM WHOLE BLOOD

2.8.1 Methods Used in Chapter 3, 4 and 8

Six mLs of venous blood was collected into preservative free EDTA (Vacuette, Greiner Labortechnik, Germany) and processed within one hour. All work was performed in a Class II Biosafety cabinet, and non-sterile surfaces were prepared with 70% ethanol. Anticoagulated venous blood was diluted 2:1 in RPMI 1640 medium (Commonwealth Serum Laboratories, Victoria, Australia) with 10% Fetal calf serum. Dilutions with phosphate buffered saline may be used with this procedure, it was felt that cell viability was improved with the use of a tissue culture medium. If viability is < 80% the medium should be fortified with fetal bovine serum (CSL, Victoria, Australia). This solution was layered onto 12 mL of Histopaque-1077, a solution of polysucrose 5.7 g / dL and sodium diatrizoate 9.0 g / dL with a density of 1.077=0.0001g/ mL (Histopaque-1077, Sigma Diagnostics, NSW, Australia: Cat No. H8889; Lot No. 64H2382). The Histopaque must be at room temperature before use to avoid limited recovery of mononuclear cells. Lower temperatures may result in cell clumping and poor recovery. The cells were centrifuged at 800 x *g* for 30 min, room temperature, with the brake off.

During the centrifugation step the erythrocytes and granulocytes aggregate by the polysucrose and rapidly sediment, while the lymphocytes and other mononuclear cells remained at the plasma - Histopaque interface. Cells were collected from the interface using a sterile Pasteur pipette. It is important during aspiration to avoid contamination of the collection with the supernatant or excess amounts of Histopaque - 1077, which may contain plasma proteins or residual granulocytes, respectively. Contaminating platelets were removed by low speed centrifugation, 600 g with no brake, initially during 3 washing steps in RPMI 1640 medium and then examined for cell viability by Trypan blue exclusion (Commonwealth Serum Laboratories, Victoria, Australia). It was recommended to perform a cell count after

the first wash to avoid any further loss of cells during the washing steps. The washing steps were often reduced to two washes if cell counts were low. Cells were resuspended in cell culture media, RPMI 1640 supplemented with 2 mmol/L glutamine, 100 IU / mL penicillin, 100 µg / mL streptomycin and 10 % foetal calf serum was used for all cell culture work.

2.8.2 Cell Counts and Assessment of Viability.

10 µL cell suspension was mixed with 10 µL of (0.25%) Trypan blue in a microcentrifuge tube at room temperature. A drop of the trypan blue/cell mixture was transferred under the coverslip of an improved Neubauer haemocytometer counting chamber (Assistent, Germany). Using light microscopy the unstained (viable) and stained (non-viable) cells were counted to obtain the total number of viable cells per mL of aliquot. This count was used to determine the number of cells in each sample and then resuspended in culture medium to give a concentration of 2 x 10^6 cells / mL.

2.8.3 Assessment of Cell Separation Using Cytospin.

Following the cell separation technique an assessment of the effectiveness of Ficoll-gradient separation samples from marsupials in Group One were examined using cytospin slides. A volume of cell suspension, approximately 150 µL, ready for culture was used. Blood smears and cytospins were stained with Leishman's stain in the standard method as described. Blood smears and differential white blood cell counts were performed where necessary. Cells in the cytospins were examined under a x100 oil immersion lens and at least 200 cells counted.

2.9 PROLIFERATION OF *P. GUNNII* PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) IN RESPONSE TO MITOGEN AND *T. GONDII* ANTIGEN STIMULATION *IN VITRO.*

2.9.1 Mitogens

Mitogens used included Lectin from *Phaseolus vulgaris* (Red Kidney Bean) (PHA) (L-9132), Concanavalin A from *Canavalia ensiformis* (Jack bean) (ConA) (C-5275), lectin from *Phytolacca americana* (Pokeweed) (PWM) (L-9379), lectin from *Lens culinaris* (lentil) (LL) (L-9267), lectin from *Arotocarpus integrifoli* (Jacalin) (JAC) (L-3515), LPS (lipopolysaccharide). All products were from Sigma Chemical Co. (St Louis, MO., USA). All were reconstituted in sterile RPMI-1640 and suitable aliquots of these stock solutions were stored at -70°C.

A crude, sonicated water-soluble preparation of *T. gondii* antigen was prepared by the Mt Pleasant Laboratory from preparations of peritoneal cavity lavage of mice (Department of Primary Industry and Water Environment, Prospect, Tasmania)

2.9.2 Culture Conditions

Standard 96-well flat-bottom microtitre plates (Greiner Labortechnik, Germany) were used for all assays, after evaluation and comparison with round and V-bottom microtitre plates. Higher proliferative responses were achieved using flat-bottom 96-well plates, often contradicting past researchers (Infante *et al.*, 1991). Responses in general by other researchers have noted that specific antigens tend to be higher with the cell crowding associated with the U-bottom geometry. However, for rapidly growing cell lines, flat-bottom wells may be superior

A single selected batch of heat-inactivated FCS was used to supplement the culture medium throughout the study reported here. 10% FCS was determined, as the optimum required to support cell proliferation without inducing excessive background stimulation. Cell cultures were incubated in a 5% CO_2 humidified

incubator at 37°C for four days and checked daily using an inverted microscope for contamination and reaction to mitogens/antigens.

2.9.3 Lymphocyte Transformation Assay

Lymphocyte proliferative responses to mitogens were measured by the incorporation of tritiated (methyl-^{3}H)-thymidine (Amersham International, England, 2.0 curies per mmol, 1.0 mCi/ mL) using a standard microculture technique. After performing the peripheral blood mononuclear cell separation, the cells were resuspended in a mixture consisting of RPMI 1640 cell culture media (Pharmacia Biotech, Sweden Cat. No. 17-091-01), 10% (v/v) foetal calf serum (FCS) (Commonwealth Serum Laboratories, Victoria, Australia), L-glutamine 2mmol 5% (v/v) (ICN Flow Laboratories, NSW, Australia), benzylpenicillin 100 U/ mL (CSL, Victoria, Australia), 100 µg/ mL streptomycin (CSL, Victoria, Australia), and made up to a final concentration of 1 x 10^6 cells / mL.

Equal volumes (100 µL) of cells were added to the wells of standard 96-well microtitre plate. Doubling dilution of mitogen were set up in a 96 well flat bottom tissue culture plate (Greiner Labortechnik, Germany) with doubling dilutions from 40% to 0.016% (v/v) of PHA and Con A, and 100% to 0.19% for PWM, JAC and LL. Cultures were performed in triplicate or duplicate depending on the number of cells, and each plate included an unstimulated control in triplicate. Depending on the quantity of cells available, further dilutions were made. As a standard procedure 180 µL of cell suspension was added to each well for a final cell concentration of 5 x 10^5 cells / mL in 200 µL. 20 µL of appropriately diluted mitogens was then added. The cells were incubated at 37°C in a 5% CO^2 humidified air incubator for 66 h, followed by a 6 h pulse with 10 µL of 1.0mCi/mL tritiated (methyl^{3}H)-thymidine. Cells were checked daily using an inverted microscope for contamination and reaction to mitogens/antigens.

2.9.4 Whole Blood Cultures

Whole blood samples (200 µL) were washed with RPMI and then diluted 1:15 with culture medium and incubated for 3 days in both round- and flat-bottom wells against Con A, PHA and PWM only.

2.9.5 Measurement of Proliferation by Uptake of Tritiated Thymidine

Cells were harvested using an automated cell harvester (Skatron Combicell harvester, 11025, Medos) onto a binder-free glass fibre filter mats (ICN Biomedicals, USA) with a particle retention size of 1.5 µM. After drying, the filter discs were transferred to 6 mL plastic scintillation vials with 2 mL of scintillant (BCS, Biodegradable Counting Scintillant; Amersham International, England). Beta emission was recorded using a 1214 Rackbeta liquid scintillation counter (LKB Wallac, Turku, Finland) and results were recorded as the sum total of the beta coincidence spectrum per minute (counts per minute; CPM).

2.9.6 Measurement of Proliferation

The mitogenic response was calculated as the mean of three triplicate or duplicate results. This method effectively minimised error due to variation of the initial cell concentration and significantly reduced interassay variability. A dose response curve was generated for all individual cultures to ensure there was no shift in the gradient of the curve.

2.10 DIRECT FLUORESCENT ANTIBODY PROCEDURE FOR DETECTION OF *GIARDIA* CYSTS AND *CRYPTOSPORIDIUM* OOCYSTS USING COMMERCIAL ANTIBODY.

- KIT: A100., AquaGlo G/C Direct, for simultaneous immunofluorescence detection of *Giardia* cysts and *Cryptosporidium* oocysts in water samples (Waterborne, New Orleans, USA) and A300Fl. Giardi-a-Glo (Waterborne, New Orleans, USA).
- FITC-labelled monoclonal antibodies – 1 mL of a 20X-concentrated solution.
- Reagent contains 0.02% w/v thimerosal as preservative and 1% bovine serum albumen as antibody stabilizer.
- Control: 1 mL of positive control mixture oocysts in 5% formalin in PBS, with approximately 25,000 cysts and 50,000 oocysts per mL.

Procedure

1) Dilute FITC-antibody reagent by 20-fold with PBS – 1X working solution.

Or blocking agent and preservative (bovine serum albumen (BSA), 1%, and/or normal goat serum (NGS 10%) and preservative (e.g. sodium azide or thirmerosal, 0.02% final conc.), to give the required 1x working solution.

- Example: 50 µL of reagent added to 950 µL of PBS/BSA/NGS to give 1 mL of working dilution OR 1 mL added to 19 mL PBS/BSA/NGS to give 20 mL working dilution etc
- Store concentrated reagent and any prepared working dilutions in a refrigerator (4 to 8°C). Do not freeze. Working dilutions are stable at 4°C for at least 1 month and probably up to 6 months or more.

2) 1X dilution/blocking buffer (PBS/1% BSA/10% NGS with 0.02% thimerosoal.

Non-specific binding sites on filters or slides containing test material should be pre-blocked with appropriate dilution/blocking buffer as described prior to applying the

working dilution of the antibody reagent. For 25 mm filters it is suggested that each filter be rinsed once with at least 2 mL of blocking solution.

3) Allow the diluted antibody reagent at least 45 min of contact time with the sample on filter or slide at room temperature or 30 min in a 37°C incubator. It is recommended that a volume of 0.5 mL be applied to 24 mm filter preparations. Incubations with slide preparations should be done in a humid chamber, e.g. Petri dish with wet lab wipes.

4) Wash filter or slide thoroughly with PBS after incubation with antibody reagent.

Rinse Protocols

1) Five 2-mL rinses with PBS or saline for 25 mm diameter filters, or one 3-minute rinse in a Coplin jar, beaker, or other suitable vessel for slides.
2) Filters may then be dehydrated with an ethanol series in glycerol and mounted on a slide with 2 % DABCO, in 90% glycerol/10% PBS (1,4-diazabicyclo (2.2.2) octane.
3) Slide preparations should be least partially air-dried and then mounted either with 2% DABCO mounting medium or with high quality fluorescence-grade immersion oil.
4) Non-specific background fluorescence may be reduced by the use of the dilution/blocking buffer described above; by the use of counterstains such as Evans Blue (0.05%) or Eriochrome Black (1 mg/mL); sometimes by diluting the working reagent another 2-fold.

2.11 GENERAL PURPOSE SOLUTIONS

2.11.1 Drabkin's solution

Potassium ferricyanide	200 mg
Potassium cyanide	50 mg
Potassium dihydrogen phosphate	140 mg
Triton X-100	1 mL
Water	1 L

2.11.2 Dulbecco's Modified PBS

Solution A

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
DDW	800 mL

Solution B

$CaCl_2$	0.1 g
DDW	100 mL

Solution C

$MgCl_26H_2O$	0.1 g
DDW	100 mL

- Mix Solutions A, B and C

NB. A, B and C can be autoclaved separately and mixed when cool.

2.11.3 FACS Fixative

40% Formaldehyde	13 mL
D-glucose	10 g
15% NaN3 in PBS	1.09 mL

- Formaldehyde, glucose and sodium azide were made up to 500 ml in PBS. The solution was stored at 4°C.

2.11.4 Hanks Balanced Salt Solution (HBSS) (Sigma Chemical Company, St Louis, USA).

HBSS powder	1 sachet (9.5g)

- HBSS (without $CaCl_2$, $MgCl_2$, $MgSO_4$, $NaHCO_3$) was reconstituted from powder using 1L of Milli-Q water, at room temperature. The solution was sterilised using a 0.22 μM filter (Millipore Corporation, USA) and stored at 4°C.

2.11.5 Neutral Phosphate Buffered Formalin (10%)

Stock solution

	Na_2HPO_4	65 g
	$NaH_2PO_4.2H_2O$	49.5 g
Or	NaH_2PO_4	35 g

- Dissolve salts separately. Make up to 2L with distilled water.

Working solution

Stock solution	400 mL
Add distilled water	1800 mL

- Lastly add 200ml of filtered formaldehyde to give a 2 L volume.

To give an immediate working solution

Na_2HPO_4	6.5 g
$NaH_2PO_4.2H_2O$	4.95 g

- Dissolve salts separately and make up volume to 900 mL with distilled water. Add 100 mL of filtered formaldehyde (40%).

2.11.6 Phosphate Buffered Saline (PBS) (20x)

NaCL	170 g
Na_2HPO_4 (anhydrous)	21.4 g
$NaH_2PO_4.2H_2O$	7.8 g

- The salts were dissolved in 750 mL Milli-Q water, over low heat using a magnetic stirrer. When cooled the solution was made up to 1 L with Milli-Q water. A 1x PBS was prepared by the addition of 50 mL of 20x PBS to 950 mL of Milli-Q water. Solutions were stored at room temperature.

2.11.7 PBS/BSA/Azide

PBS- 2% BSA - 0.1% Azide was prepared as follows:

1x PBS	1000 mL
BSA	20 g
NaN_3	1 g (sodium azide)

Sodium azide and BSA were placed on top of the PBS and left to dissolve for several hours (overnight). The solution was stored at 4°C.

2.11.8 Red Blood Cell (RBC) Lysis buffer

10 mM Potassium bicarbonate	0.50 g
0.15 M Ammonium chloride	4.01 g
0.1 mM EDTA	18.6 g
5% FCS	25 mL

- All ingredients were dissolved in Milli-Q water and the volume made up to 500 mL. RBC lysis buffer was stored at and used at 4°C.

2.11.9 RPMI (Complete)

RPMI-1640	500 mL
Foetal calf serum (FCA)-heat inactivated	50 mL
L-glutamine, 100x	5 mL
Penicillin Streptomycin	1 mL
Sodium pyruvate	5 mL
2-mercatpoethanol (0.5 μM)	0.5 mL

- Solutions were mixed and sterility maintained. Complete RPMI was used for maintaining cells in a viable, functional state for dye efflux and accumulation assays. Stored at 4°C.

2.11.10 Trypan Blue (Chroma-Gesellschaft, Schmid and Company Germany)

Trypan blue powder	0.25 g
Milli-Q water	100 mL

- Sterilised through 0.22 μM millipore filter.

2.11.11 Turk Diluting Fluid

Glacial acetic acid	1 mL
Gentian violet (1% aqueous)	1 mL
Distilled water	100 mL

- Filter solution before use.

Chapter 3. Experimental Toxoplasmosis in *Perameles gunnii*

3.1 INTRODUCTION

Since the introduction of cats with European settlement over 200 years ago, or perhaps even earlier with seafarers and shipwrecks (Wagner, 1997), Australia's native fauna have suffered severely from their presence. Predation by feral and domestic cats are known to affect population numbers of *Perameles gunnii* in Tasmania (Mallick, 1997b), but few have analysed the role of cats and their significance in disease transmission, especially of toxoplasmosis.

Members of the cat family (Felidae) are the only definitive hosts of *Toxoplasma gondii* (Dubey, 1994) and as such, domestic and feral cats are responsible for transmitting this disease to a wide range of naïve intermediate hosts. Serological examination of feral cats in Tasmania reported the prevalence of toxoplasmosis to be as high as 50% (Milstein and Goldsmid, 1997). There are many reports of subclinical infection or overt disease in captive and free-ranging Australian marsupials (Obendorf and Munday, 1983; Canfield *et al.*, 1990a; Lenghaus *et al.*, 1990; Obendorf and Munday, 1990; Bourne, 1997; Miller *et al.*, 2000). Although Australian marsupials are very susceptible, and recovery from clinical disease is rare, an appreciable number do survive infection, as shown by seroconversion and the presence of *T. gondii* tissue cysts at post-mortem examination of animals dying of other causes (Pope *et al.*, 1957; Cook and Pope, 1959; Munday, 1972a; Kollias *et al*, 1976; Johnson *et al.*, 1988). An infected intermediate host normally develops immunity as a consequence of infection with *T. gondii* protecting it against subsequent challenge, but the protozoan can be an important pathogen in immunologically vulnerable hosts (Wong and Remington, 1993). Some episodes of acute disease may be the result of stress on animals with chronic *Toxoplasma* infection (Obendorf and Munday, 1983).

Toxoplasmosis has been reported in wild bandicoots in Victoria and Tasmania, when often they show no clinical signs or significant antibody levels before their sudden death (Pope *et al.*, 1957; Obendorf and Munday, 1990). The first reports of a central nervous system disease in Tasmanian *P. gunnii* were in 1984 (Obendorf and Munday, 1990). Several bandicoots in southern Tasmania were observed showing signs of incoordination, erratic staggering movements, apparent blindness, unnatural daytime activity and eventual death. The only gross findings at necropsy examinations were poor body condition, absence of ingesta and generalized pulmonary consolidation (Obendorf and Munday, 1990). Histological examination of the brain revealed focal areas of non-suppurative meningo-encephalitis characterised by tissue necrosis infiltration of inflammatory cells and the presence of protozoan cysts typical of *T. gondii*. *T. gondii* cysts were detected in the lungs in association with congestion and oedema. Non-suppurative myocarditis in association with *T. gondii* cysts was described, leaving no doubt that *T. gondii* was the pathological agent in the death of these animals.

The prevalence of *T. gondii* antibody in free-ranging Tasmanian *P. gunnii* populations conducted by Obendorf *et al.*, (1996), concluded that approximately 95% of animals were seronegative and therefore previously unexposed, but more significant, the few individuals that had detectible antibodies were never retrapped or found and were thus assumed to have died. The conclusion drawn was that infected bandicoots did not survive primary infection with *T. gondii,* and unlike other marsupials, the disease did not appear to result from reactivation of a latent infection. This conclusion prompted the current experimental investigation, to observe the course of the disease and formation of antibodies in *P. gunnii* following experimental oral inoculation with a known number of viable *T. gondii* oocysts.

Determining the direct source of toxo-infection is difficult due to the ubiquitous nature of the parasite, but investigations on the epidemiology in Tasmania confirmed

the importance of the cat-sheep cycle in the perpetuation of this parasite (Munday, 1970, 1975; Hartley and Munday 1974). It was noted that the high prevalence of toxoplasmosis in sheep reflected the occurrence of toxoplasmosis in wallabies inhabiting sheep-grazing areas of Tasmania. This implicated both feral and domestic cats from adjacent farms and urban areas as the source of contamination (Lenghaus *et al.*, 1990).

In general, marsupials may acquire infection by exposure to a number of sources. Vegetation contaminated with cat faeces is a probable reservoir of infection for wild herbivorous marsupials (Patton *et al.*, 1986) while contaminated grains and hay pose the greatest threat to animals held in captivity. Outbreaks of toxoplasmosis in carnivorous animals have been linked to meat with tissue cysts fed to animals (Borst and van Knapen, 1984; Dickson *et al*, 1983). Water and soil contaminated with the oocysts derived from cat faeces is another likely source of infection. For omnivorous and insectivorous marsupial species such as bandicoots, a probable source of infection is by ingestion of annelids (earthworms) and arthropods that contain *T. gondii* oocysts in their digestive tract or carry the parasite on their body surface. The arthropods themselves acquire the oocyst from ingesting contaminated soil, plant material or faeces (Frenkel *et al.*, 1975; Ruiz and Frenkel, 1980, Obendorf and Munday, 1990). Based on dietary evidence available, it was speculated that *P. gunnii* acquire infection through ingestion of these soil-associated oligocheates (earthworms) and arthropods containing infective stages of *T. gondii.* The current investigation presents a series of experimental feeding studies undertaken to assess the role of earthworms in the transmission of toxoplasmosis to *P. gunnii.*

3.2 MATERIALS AND METHODS

3.2.1 Experimental Animals

In 1995, four adult *P. gunnii* (1 female, 3 males) were captured from a wild population at Kingston and four adults (3 females; 1 male) were trapped in the Huon Valley. All animals weighed between 700-790 g. Trapping was in accordance with established guidelines and protocols of Parks and Wildlife Service permit to take protected wildlife (Hobart, Tasmania, Australia; permit numbers 95249 and 95256) and outlined in Chapter 2; 2.1, 2.1.2. The animals were captured using wire cage traps (Mascot Wire Works, Enfield, New South Wales, Australia) and housed in the Central Animal House at the University (Hobart, Tasmania; Animal Ethics Committee approval number 94010). Each animal was initially placed in single-animal holding cages with a nest box. All animals were allowed to acclimate to their environment and food source for a month prior to experimental work. Animals were screened for blood parasites and a profile of faecal endoparasites recorded. *Toxoplasma* serology was performed to exclude animals with *T. gondii* antibodies. After this period all animals were transferred from the original holding pens to individual sterilized enclosures.

3.2.2 Animal Diet

The diet formula was adapted from notes by Tasmania's Parks and Wildlife Service, Department of Primary Industries, Water and Environment (DPIWE) (Chapter 2; 2.1.4). Care was taken to exclude any possibilities of extraneous infections at all stages of food handling and preparation. Water was provided *ad libitum*.

3.2.3 General Maintenance

At certain stages of the experimental work each bandicoot was held in a separate holding pen (2.5 x 1.0 m in floor area, with 0.6 m walls) made from freshly milled timber; the floor was clean pine sawdust. Each pen contained a wooden hutch with

shredded paper as nesting material; pens were covered with shade cloth to prevent escape and reduce distress. Food and water was provided *ad libitum*. All materials were cleaned and sterilized to exclude any contamination from cat faeces or exposure to *T. gondii*. Animals were regularly weighed and handled prior to experimental work, in preparation for repeated testing and blood sampling with minimal stress. Sawdust and shredded paper was changed regularly and incinerated.

3.2.4 *Toxoplasma gondii* oocysts

The P89 / VEG strain oocysts were transported in 2% H_2SO_4 in water, and were stored at 4°C until diluted (kindly provided by Dr J.P. Dubey, USDA, Beltsville, Maryland, USA).

3.2.5 Animal Inoculation: Experiment 3.1

Two bandicoots (No 01 and 03) were inoculated orally with *T. gondii* oocysts of the P89 / VEG strain. This strain was selected for its standardised virulence for mice, one oocyst being lethal to mice by any route of administration (Dubey, 1995). The concentration of oocysts was determined by direct microscopic counts using a Neubauer haemocytometer counting slide; 100 cysts were given *per os* to each animal in saline suspensions formulated at concentrations of 100 cysts per mL. One ml of suspension was deposited directly in the oesophagus with the aid of a canula.

The inoculated marsupials and control animals (No. 04 and 06) were housed in sterilized stainless steel wire cages. Bedding and excreta were collected for 5 days post infection from the cages. All material was incinerated to ensure oocysts that may have passed unexcysted in faeces were killed. At 5 days post-infection animals were transferred to individual holding pens; their diet remained the same with water *ad libitum*; each day the bandicoots were observed for any clinical changes and behaviour.

3.2.6 Earthworms and Soil Conditions: Experiment 3.2

Earthworms used were commercially reared Red earthworm *Lumbricus rubellus* and the Blue earthworm *Perionyn excavatus* (All State Worm, P/L, Adelaide, South Australia). Soil treatment and earthworm maintenance is described in Chapter 2; 2.6.

Five hundred artificially-reared *Lumbricus rubellus* and *Perionyn excavatus* earthworms were initially prepared by passage through two changes of the sterile soil mix over a 2 day period. Every 24 hours the earthworms were carefully sieved and washed in 0.9% NaCl solution and transferred to fresh soil mix. When fully purged, the worms were divided equally between two plastic boxes containing fresh soil. The soil was enriched with a protein mix of eggs and sterile milk powder once every 2 weeks to provide nutrient for the worms. One group was maintained in uninfected soil as the control group, the other group was placed in soil infected with *T. gondii* oocysts.

A suspension of 1.75 x 10^6 *T. gondii* oocysts (P89/VEG strain) was mixed in 25 mL of distilled water and dispersed onto the soil surface. The sample was delivered with a hand held spray unit some 200 mm above the soil to reduce aerosols. The surface was gently mixed and left for one week to ensure that the worms were fully exposed. Soil samples tested for the presence of oocysts using a direct fluorescent antibody test proved to be positive for *T. gondii* oocysts. One box remained unadulterated. The infectivity of the oocysts was demonstrated by directly inoculating bandicoots orally with approximately 100 oocysts, Experiment 3.1.

3.2.7 *P. gunnii* Infection with Earthworms: Experiment 3.2

After 7 days, earthworms from the soil containing oocysts were carefully removed and washed in normal saline to remove excess organic matter and soil debris. Twenty five g aliquots of worms were added to the daily food ration of each of two

captive *P. gunnii* (No. 07 and 08) on day 8, with all other meat products being excluded. These animals were fed another 25 g meal on day 9. Remaining washed earthworms from this box were transferred into a new box containing freshly autoclaved soil. Over a 4 to 5 day period the worms were transferred through four changes of fresh soil every 18 to 24 hours as described above during the purging cycle. Once completed the worms were washed and two serves of 25 g aliquots were added to food given to each of another two bandicoots (No. 02 and 05) for one or two days again excluding other meat products. An additional two bandicoots (No. 04 and 06) were each fed 25 g of earthworms derived from the control box (i.e. containing sterilised soil only).

3.2.8 Blood / Serum Collection / Observations

Blood samples were collected from a peripheral ear vein of all animals while the animals were restrained. The perimeter of the ear was sterilized with alcohol and the vessels dilated by light massaging and a marginal blood vessel was lanced. Method for blood film examination was described in Chapter 2; 2.2. For serum collection, 0.5 mL of blood was collected into Microtainer serum separating tubes (Microtainer: Becton Dickinson & Co., Rutherford, New Jersey, USA) with a gel interface. On each sampling session at 0, 6 and 13 days post infection (DPI) bandicoots were weighed, observed and their body temperature taken by digital thermometer inserted into the rectum.

3.2.9 Toxoplasmosis Direct (DAT) and Modified (MAT) Agglutination Tests (Chapter 2; 2.5)

All animals selected for the study tested negative for *T. gondii* antibodies prior to the commencement of experimental work. Two serological tests were used to screen the sera for *T. gondii* antibody. The Direct Agglutination Test (DAT) and Modified Agglutination Test (MAT) were performed using the commercial kit Antigene Toxo-AD and microtiter plate reagents (bioMerieux SA, Marcy-l'Etoile, France). The

tests were performed at Mt Pleasant Laboratories (Department of Primary Industries, Water and Environment, Tasmania) and results recorded as described by Obendorf *et al.*, (1996)

Formalin-treated *T. gondii* tachyzoites used as the antigen were derived from wild infected *P .gunni* and cultured *in vivo* in mice. In the DAT, a 25 µL serum sample was initially tested at 1:8 and titrated in two-fold serial dilutions to 1 : 4096. In the MAT, a 25 µL serum sample was initially mixed with 25 µL of 0.2 M 2-mercaptoethanol (2-ME) (BDH, Poole, England) in phosphate buffered saline (PBS) before being similarly tested in serial dilutions. All animals were seronegative prior to the commencement of experimental work. An additional blood sample was taken at necropsy.

3.2.10 Histopathological Examination (Chapter 2; 2.4)

Selected organs and tissues from animals that died were fixed in 10% buffered formol-saline; histological specimens (prepared in the Discipline of Pathology, University of Tasmania) were embedded in paraffin, sectioned at 4 µM and stained with haematoxylin and eosin (H&E) and microscopic examination. Duplicate sections were prepared on Chrome Alum slides for immunohistochemistry.

3.2.11 Immunohistochemistry (Chapter 2; 2.4)

This method was adapted from a modified procedure used at Mt Pleasant Laboratories, (Department of Primary Industries, Water and Environment, Tasmania) (1:500 in PBS, 1 µL in 50 mL PBS). Duplicate sections prepared on Chrome Alum slides were stained using avidin-biotin peroxidase complex (ABC) from the commercially available Vectastain AB Rabbit IgG kit (Vector Laboratories, Bulingame, California, USA). Sigma Fast DAB (Sigma Chemical Company, St. Louis, Missouri, USA) was used to simplify the final preparation of slides. Rabbit-anti *T. gondii* serum was used as the secondary antibody (kindly supplied by

Department of Primary Industries, Water and Environment, Tasmania). Positive tissue controls included brain with lesions of *T. gondii* infection from *P. gunnii*, a Bennett's wallaby (*Macropus rufogriseus*) and a common wombat (*Vombatus ursinus*) (DPIWE No. 93/4534, 95/2026, 93/6268). Negative tissue controls included tissue sections from *P. gunnii* with no microscopic pathology (DPIWE No. 95/7969; 96/8090).

3.2.12 Lymphocyte Transformation Assay

The lymphocyte transformation assay was used to measure the proliferative responses of *P. gunnii* lymphocytes in response to mitogens and *T. gondii* antigen stimulation. The methods are described in Chapter 2; 2.8 and 2.9

3.3 RESULTS

3.3.1 Assessment of *P. gunnii* Pre- and Post-Infection

P. gunnii (No 01 and 03) orally dosed with P89 / VEG oocysts died 15 and 17 days post infection (DPI) (Table 3.1). The presence of *T. gondii* infection was confirmed by gross and microscopic examination. Weight gain and loss was recorded in all animals post infection, with animal No. 01, recording a loss of 40 g post infection and No. 03 only 10 g (Table 3.2). The body temperature of animals post-infection was particularly variable. Maximum body temperatures over 35°C were recorded on Day 2, but for animal No. 01, temperatures were extremely variable with a minimum of 34.0 °C recorded three times during the course of infection (Table 3.3a). No. 01 recorded an average 34.0 $\pm$ 0.59 °C body temperature, the maximum of 35.9 °C recorded on Day 2. Similarly, No. 03 recorded an average 35.1 $\pm$ 0.22 °C, with the maximum body temperature of 35.5 °C recorded on Day 2 (Table 3.3b) At 10 DPI both inoculated animals showed abnormal movement and behaviour outside their nest boxes during daylight hours and an increase in water intake. Loss of appetite was noted three days prior to death.

Experimental Animals	*P. gunnii* No #	Survival Days Post Infection	DAT: MAT
Oral inoculation	01 male	15	256 : 0
Oral inoculation	03 female	17	64 : 0
Earthworm	07 male	14	16 : 0
Earthworm	08 female	11	64 : 16
Earthworm (purged)	02 male	-	Neg
Earthworm (purged)	05 female	-	Neg
Control	04 female	-	Neg
Control	06 female	-	Neg

Table 3.1 Serology results of *P. gunnii* following experimental infections with *Toxoplasma gondii.*

DPI	*P.gunnii* No.01	No.02	No.03	No.04	No.05	No.06	No.07	No.08
1	870	800	910	860	850	620	850	890
2	830	770	890	840	850	600	820	840
3	840	770	910	840	860	600	860	850
4	840	790	920	860	890	610	**880**	860
5	830	790	940	860	910	620	860	800
6	850	790	870	900	890	660	820	800
7	830	790	870	900	930	660	830	800
8	840	770	940	900	930	680	800	810
9	830	780	930	890	860	630	810	805
10	830	790	900	890	860	640	810	810
11	840	770	890	930	870	640	805	
12	840	780	900	920	860	620	810	
13	840	800	920	860	850	675	800	
14	830	800	900	880	850	695	**Earthworms**	
15	830	800	900	920	860	715		
	***T. gondii* / os**	**Control**	900	**Control**	**Control**	**Control**		
			***T. gondii*/os**					

Table 3.2 Recorded weight (g) distribution of *P. gunnii* (No. 01-08) during acclimatisation and *Toxoplasma gondii* oocysts *per os.*, and earthworm feeding experimentation.

DPI	No. 01	No.02	No.03	No.04	No.05	No.06	No.07	No.08
1	35.2	35.1	35.2	35.4	35.8	35.2	35	35.1
2	35.9	34.5	35.5	35.7	35.4	36.1	35.5	34.9
3	35.0	34.8	35.1	35.2	35.1	35.1	34.5	34.9
4	34.9	35.3	34.9	34.0	35.7	35.4	35.0	34.9
5	34.0	34.9	34.8	35.5	35.7	35.7	35.0	35.0
6	35.5	35.5	34.8	35.0	35.0	35.0	35.1	35.2.
7	34.8	35.2	34.9	34.9	34.9	34.8	34.9	35.1
8	35.1	34.9	35.3	35.0	35.0	35.1	35.0	35.0
9	35.2	35.1	34.8	35.3	35.0	35.4	35.4	34.9
10	34.0	35.7	35.3	34.9	35.1	35.2	35.7	35.0
11	35.5	35.7	34.9	35.5	34.9	35.0	35.0	
12	35.0	35.0	35.0	35.2	35.0	34.8	35.1	
13	34.0	34.9	34.9	35.0	35.0	35.1	35.1	
14	34.5	35.2	35.0	35.3	34.9	35.0	Earthworm	
15	*T. gondii* per os	34.9	35.3	34.9	35.0	35.0		
16		35.1	35.3	35.0	35.0	35.1		
		Control	*T.gondii* per os	Control	Control	Control		

Table 3.3a Body temperature of *P. gunnii* days post-infection (DPI) with *Toxoplasma gondii* oocysts *per os*., and feeding of infected earthworms.

Temperature °C in *P. gunnii* Post-Infection with *T. gondii*				
P. gunnii	Mean	Std Dev	Min	Max
No. 01	34.9	0.59	34.0	35.5
No. 03	35.1	0.22	34.8	35.5
No. 07	35.1	0.29	34.5	35.7
No. 08	35.0	0.11	34.9	35.2
Control *P. gunnii*				
No. 02	35.1	0.32	34.5	35.7
No. 05	35.2	0.31	34.9	35.8
No. 04	35.1	0.39	34.9	35.7
No. 06	35.2	0.33	34.8	35.7

Table 3.3b Body temperature of *P. gunnii* days post-infection (DPI) with *Toxoplasma gondii* oocysts *per os*., and feeding of infected earthworms.

P. gunnii (No. 07 and 08) fed on earthworms derived from soil containing oocysts respectively lost 80 g and 140 g in body weight and died 14 and 11 days after the initial earthworm feed (Table 3.1). Both animals were active during daylight hours and water intake increased two to three days prior to death. Body temperature was variable with an average 35.1 ± 0.29°C and 35.0 ± .11°C recorded for each animal respectively (Table 3.3b). Animal No. 08 displayed incoordinated movements and an inability to locate its shelter box at 9 DPI. No significant increase in body temperature was noted in either animal during the course of the investigation.

P. gunnii (No. 02 and 05) were fed on earthworms which had been initially exposed to *T. gondii* oocysts but were then purged in fresh soil, before feeding to the bandicoots. These animals remained clinically normal with no behavioural changes observed. Control animals (No. 04 and 06) maintained their appetite throughout the experiment and no behavioural or physical changes were observed.

3.3.2 Serological Response to Experimental Infection with *Toxoplasma gondii.*

Serology results from the experimental infections are summarised in Table 3.1. Both orally inoculated bandicoots (01 and 03) tested for antibodies using Direct Agglutination Test (DAT) had a titre at the time of death of 1: 256 and 1: 64 but tested negative with the Modified Agglutination Test (MAT). At 6 and 13 DPI, *P. gunnii* No. 01 had a DAT titre of 1:256 while No. 03 was negative on both days. Both agglutination tests used in this experiment were utilised in past programs to diagnose *T. gondii* infection in humans, domesticated animals, and marsupials (Obendorf *et al.*, 1996). These results were classified according to data set by Obendorf *et al.*, (1996) and based on the results of confirmed toxoplasmosis cases of *P. gunnii* in the wild (range: DAT 256 to 64,000; MAT 64 to 64,000). In summary, the results were considered negative when the DAT titre was <64 and no reaction in MAT. Results were considered suspicious when the DAT titre was ≥64 and no reaction in MAT, and positive when both DAT and MAT titres were ≥64.

P. gunnii, No. 07 and 08 (fed on oocyst-infected earthworms), recorded low agglutination titres detected with DAT (1: 16; 1: 64) while animal No. 08 recorded a low MAT titre (1: 16). Animals No. 02 and 05, fed on purged worms, remained clinically healthy and did not develop antibodies during the 6 weeks after the experiment began. Similarly, control animals No. 04 and 06 fed on worms maintained in autoclaved soil remained healthy and seronegative over the same period. Both control animals remained seronegative throughout the experiment and were released back into their native habitat.

3.3.3 Pathology: Gross Findings

Notable necropsy findings in all four infected *P. gunnii* included the presence of excessive blood-tinged abdominal fluid, enlarged mediastinal lymph nodes, and splenomegaly. All had severe pulmonary congestion, oedema, and focal consolidation with areas of haemorrhage. The heart was grossly enlarged and the liver was enlarged and mottled in appearance, the latter with a distinct lobular pattern to the parenchyma. Regions of the gastrointestinal tract, including stomach and small intestine, were characterised by small intestine serosa and petaechial haemorrhages. Oedematous pale yellow mesentery was common, with enlarged lymph nodes scattered throughout. Segments of small intestine were often yellow-beige. An excessive amount of serous abdominal fluid was present in both animals, but this was especially marked in animal No. 03.

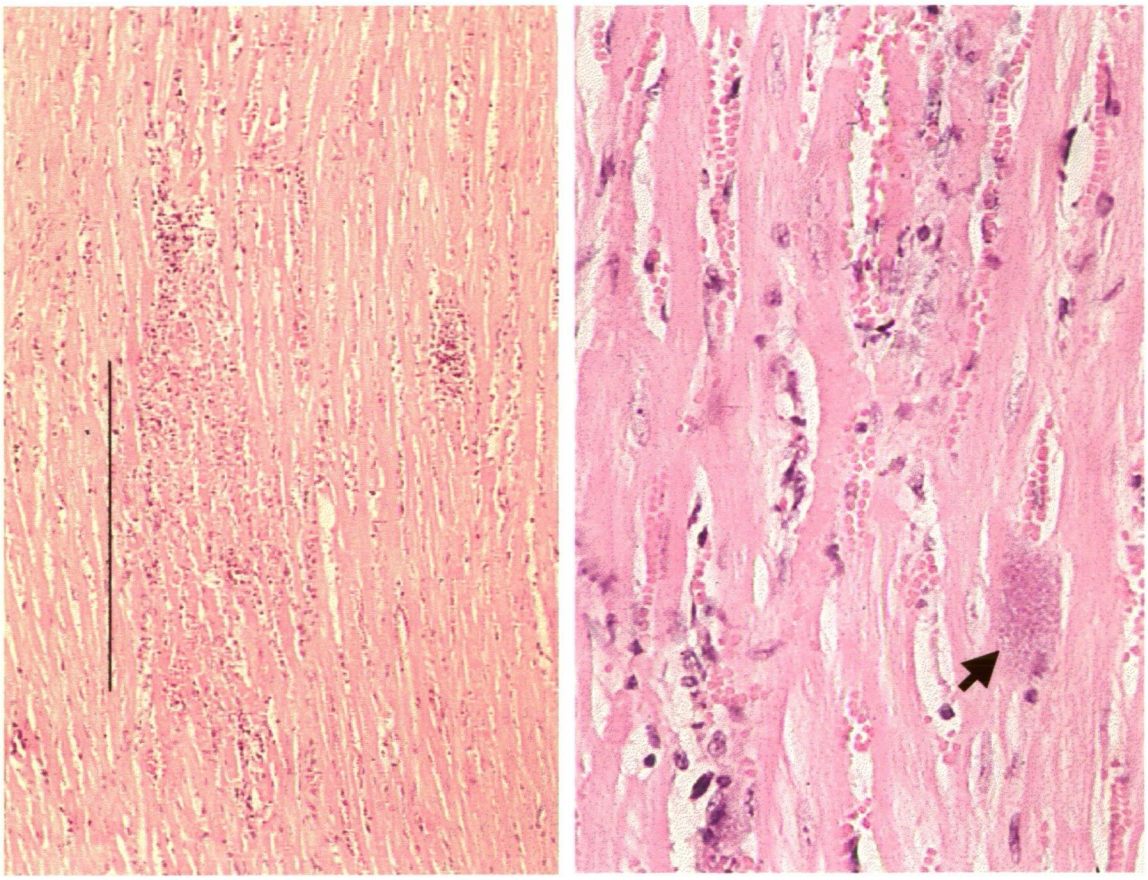
3.3.4 Pathology: Microscopic Findings

Microscopic lesions were found mainly in visceral organs of all four infected *P. gunnii.* Control animals No. 02 and 05 were euthanased in good health with no gross or histological findings suggesting the presence of *T. gondii.*

In animals No. 01, 03, 07 and 08, extensive cell necrosis associated with acute inflammation was apparent in many tissues and organs, the most severe histopathological changes being seen in heart muscle, liver, lung, small intestine, lymphoid follicles of the spleen and lymph nodes, pancreas and skeletal muscle. Immuno-staining specific for *T. gondii* trophozoites was used to identify individual organisms or cyst-like aggregates within several types of host cell and in close association with areas of general cell necrosis.

The heart contained focal areas of non-suppurative interstitial inflammation and myonecrosis with infiltrates of mononuclear cells or histiocytes (Figure 3.1). Cyst-like aggregations of *T. gondii* were often seen with H&E (Figure 3.2). The liver had multifocal areas of hepatocellular necrosis (Figures 3.3 and 3.4). The lungs had extensive interstitial pneumonia associated with cellular infiltrates into alveolar tissues, acute inflammatory changes and areas of focal fibrinous necrosis. *T. gondii* induced pneumonitis can be seen in Figure 3.5. Acute inflammation was commonly seen in the submucosal layers of the small intestine. Small patches of *T. gondii* cysts were scattered in the submucosal layers and represented in Figure 3.6. Acute inflammation and cell necrosis associated with tachyzoites and cysts was seen in the skeletal muscles and pancreas. Muscle degeneration was commonly associated with diaphragm and skeletal muscle (Figure 3.7 and 3.8).

Spleen showed areas of lymphoid depletion (Figure 3.9) with extensive areas of infiltration with mononuclear cells (Figure 3.10 (a)). Cysts of *T. gondii* were commonly identified (Figure 3.10 (b)). Lymph nodes showed moderate to severe necrosis of the lymphoid follicles with aggregates of *T. gondii* tachyzoites, sometimes in the form of close clusters, in areas of necrosis. Numerous tachyzoites and cysts were seen in association with these lesions. These stained positively in the avidin-biotin peroxidase reaction, as seen in lymphoid sections in Figures 3.11 (a) and (b).



Toxoplasma gondii cardiac lesions of experimentally infected *P. gunnii.*

Figure 3.1. Focal areas of mononuclear infiltration in cardiac muscle.

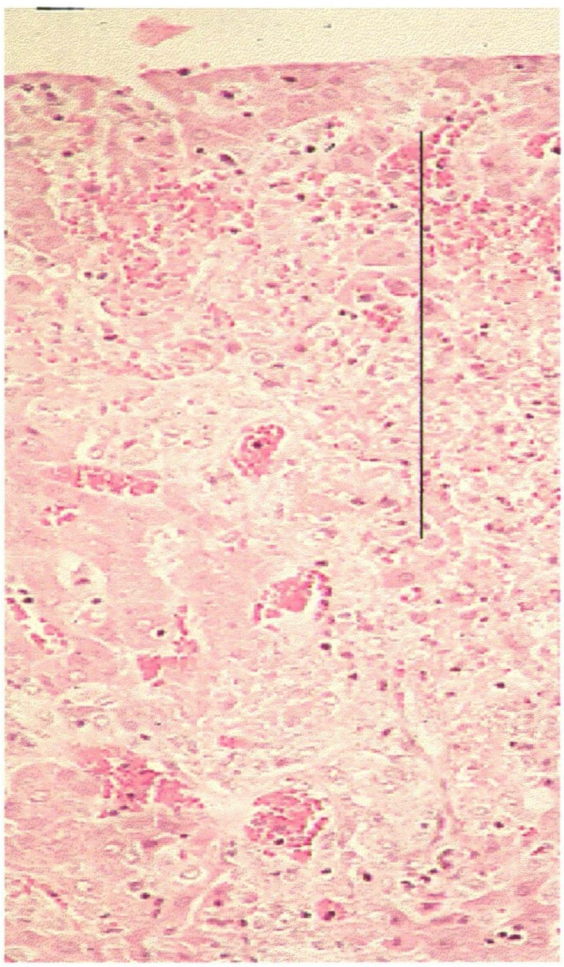
Stain - H & E

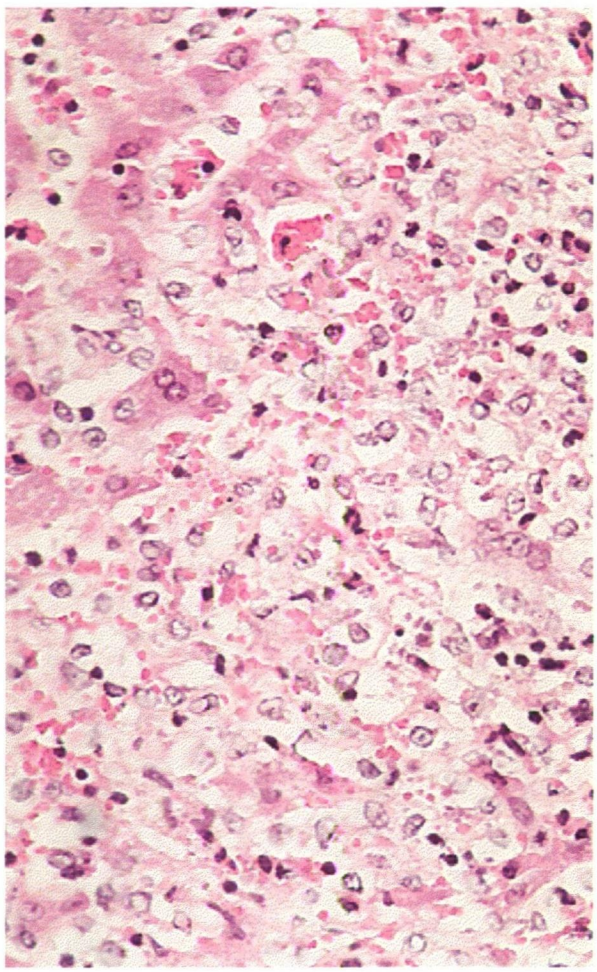
Magnification - X 100

Figure 3.2. Cardiac muscle necrosis with a cyst-like aggregation of *T. gondii.* (arrow)

Stain - H & E

Magnification - X 250





Toxoplasma gondii lesions in liver of experimentally infected *P. gunnii.*

Figure 3.3. Free zoites/cysts scattered across section of liver.

Stain - H & E

Magnification – X 60

Figure 3.4. *T. gondii* induced necrosis in liver.

Stain - H & E

Magnification – X 300

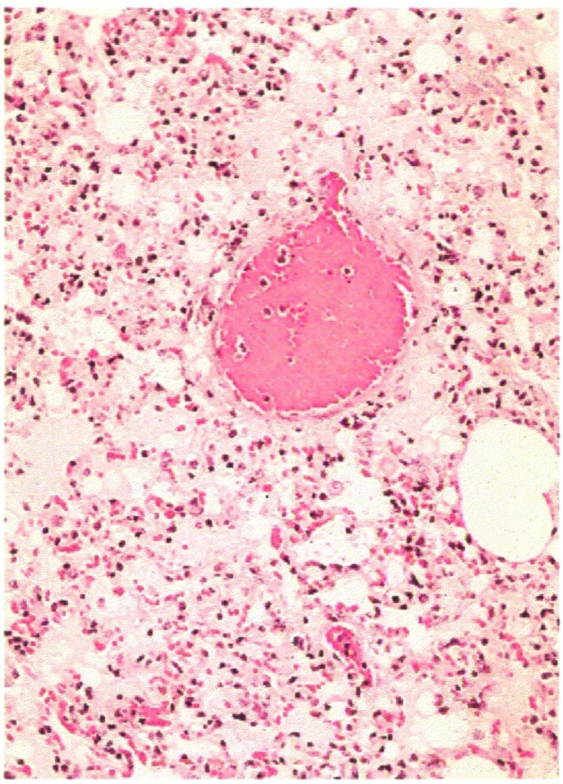


Figure 3.5. Pneumonitis in *P. gunnii* associated with *T. gondii.*

Stain - H & E

Magnification – X 150

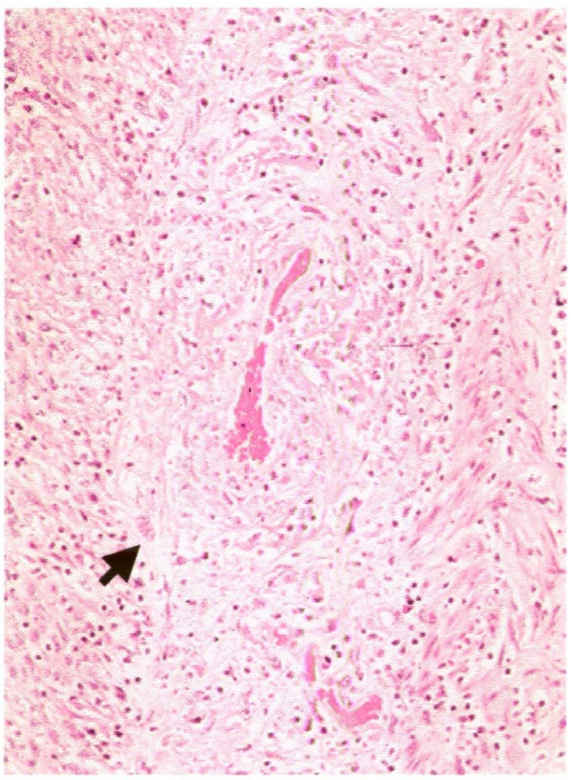
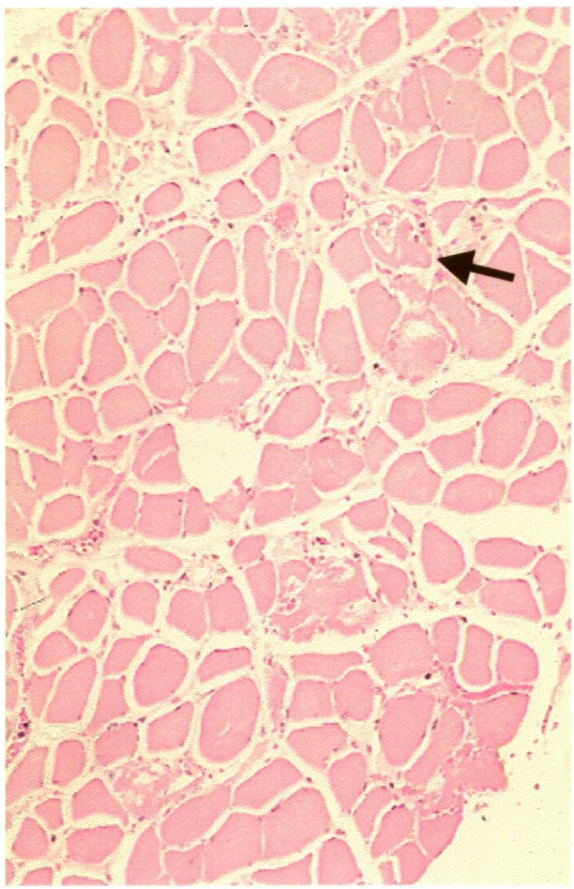
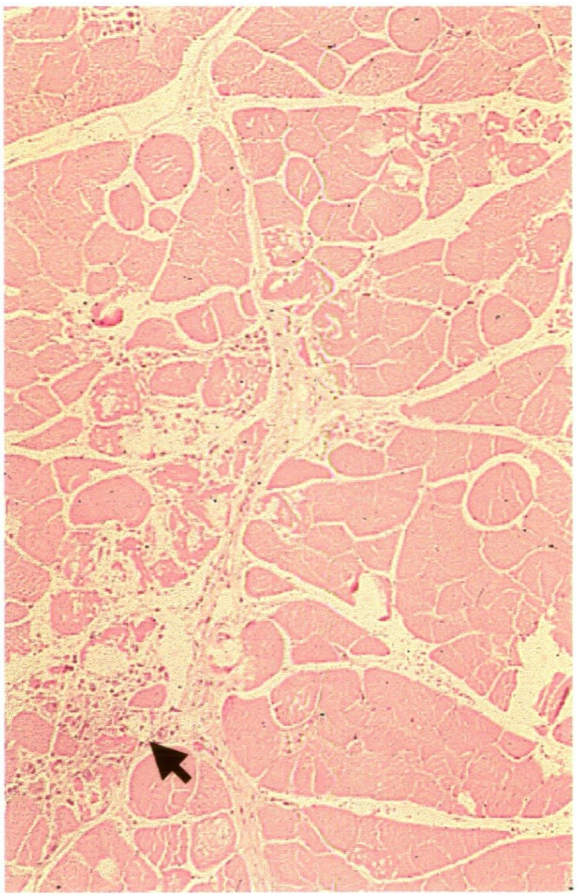


Figure 3.6. Development of *Toxoplasma gondii* cysts (arrow) in lesions of the small intestine wall of *P. gunnii* with infiltration of mononuclear cells into tissue.

Stain - H & E

Magnification – X 200





Muscle degeneration as a result of *Toxoplasma gondii* in experimentally infected *P. gunnii*.

Figure 3.7. Muscle degeneration in section of diaphragm of *P. gunnii* following *T. gondii* infection (arrow).

Stain - H & E

Magnification – X 100

Figure 3.8. Muscle degeneration of smooth muscle in *P. gunnii* (arrow).

Stain - H & E

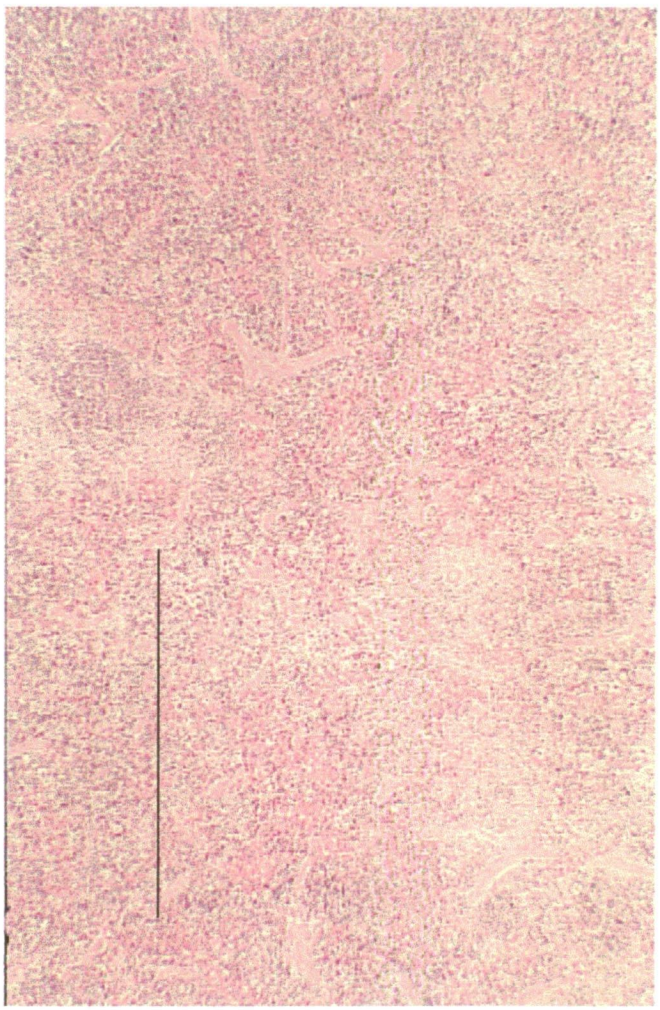
Magnification – X 100

Figure 3.9.

Toxoplasma gondii lesions in the spleen of experimentally infected *P. gunnii*. Spleen shows areas of lymphoid depletion.

Stain - H & E

Magnification – X 60



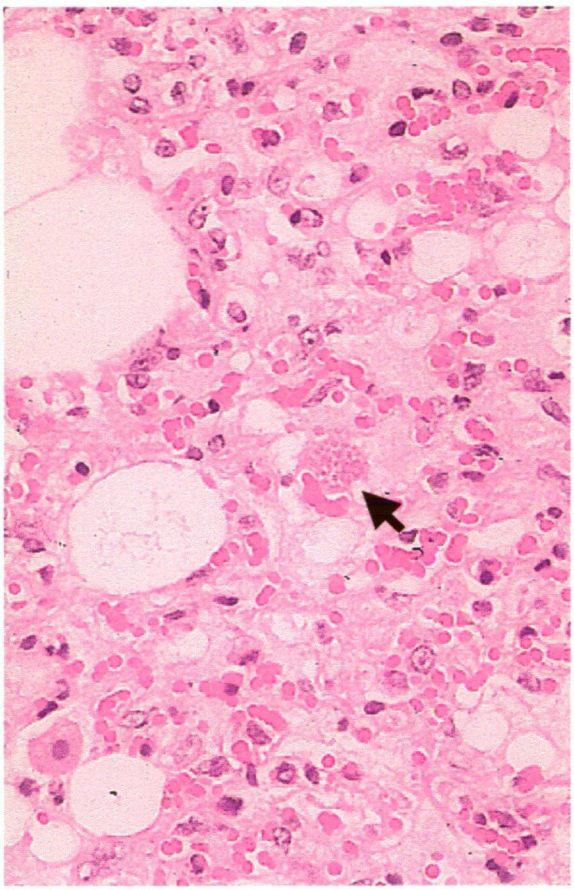


Figure 3.10 (a). *Toxoplasma gondii* lesions in spleen of experimentally infected *P. gunnii* with infiltration of mononuclear cells. Section shows cyst of *T. gondii* (arrow).

Stain – H & E.

Magnification - X 400

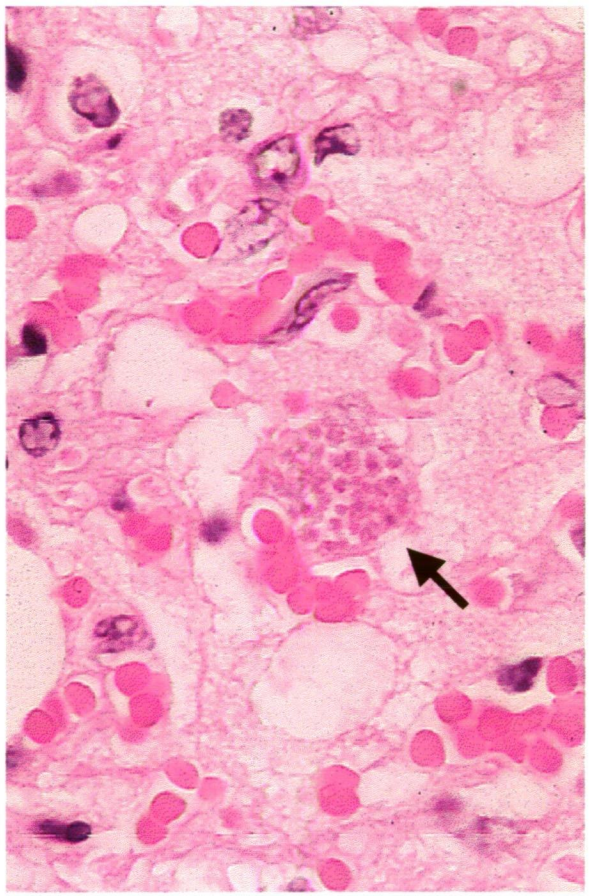


Figure 3.10 (b). Section of spleen at higher magnification showing cyst of *T. gondii*. (arrow).

Stain – H & E.

Magnification - X 850.

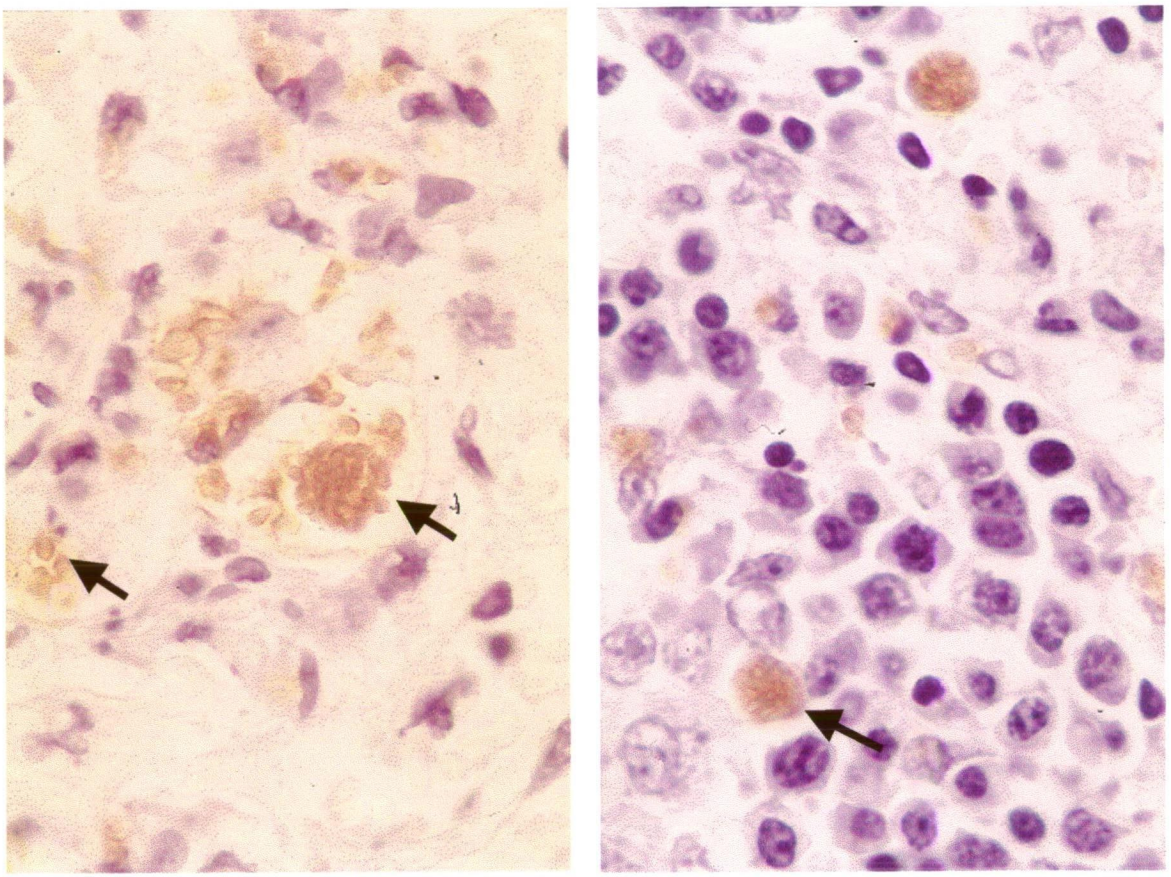


Figure 3.11 (a) and 3.11 (b). A collection of *Toxoplasma gondii* in necrotic lymphoid tissue following immunoperoxidase staining (arrows).

Stain - H & E

Magnification – X 1000

3.4 DISCUSSION

The first point of note in considering these results is that in both instances where overt toxoplasmosis occurred, the *P. gunnii* concerned were initially young healthy adults, and representatives from a 'normal' bandicoot population of Tasmania. It must also be considered that clinical manifestations of toxoplasmosis can depend on a number of factors, including the strain of the parasite, the dose size, route and form of the infecting parasite (Frenkel, 1973; Dubey and Carpenter, 1993). Similarly, neonates in most species have a reduced immune competence compared with older animals of the same species, and are generally more susceptible to toxoplasmosis (Dubey and Frenkel, 1972; Frenkel, 1990; Lloyd, 1995).

In this investigation, bandicoots given *T. gondii* oocysts *per os.*, died at 15 and 17 DPI respectively, with no detectable antibodies in the terminal stages of the disease. Gross lesions noted in these bandicoots were similar to those of experimentally infected Tammar wallabies (*Macropus eugenii)* (Reddacliff *et al.*, 1993). The histopathology was characterised by extensive cellular necrosis, acute inflammation predominated by neutrophil infiltrations and the presence of large numbers of tachyzoites either as dispersed single organisms or in close aggregates or cysts. The generalised cell necrosis of lympho-reticular tissues were possibly responsible for the weak humoral antibody response in the infected *P. gunnii.* The severity of microscopic changes seen in many organs and tissues in association with proliferation of *T. gondii* would suggest that the dose and strain of *T. gondii* administered was extremely pathogenic for *P gunnii.* An earlier attempt to infect bandicoots with a lower dose of 10 oocysts of this strain was unsuccessful in producing clinical manifestations or antibody production. Due to the restrictions imposed on the study by the fact *P. gunnii* is listed as a vulnerable species, repeat experimentation using additional animals inoculated with smaller numbers of oocysts was not possible.

The antemortem diagnosis of toxoplasmosis and the distinctions between infection and active disease has often been difficult in marsupials (Miller *et al.*, 2000). The direct agglutination test (DAT), was first developed by Fulton and Turk, (1959) and consequently modified by Desmonts and Remington, (1980) for multiple species. The modification included the addition of 2-mercaptoethanol to eliminate IgM reactivity and the resulting assay, the modified agglutination assay (MAT), was used to measure IgG antibodies to *T. gondii.* Both these assays have successfully been used to monitor the development of specific *T. gondii* antibody in marsupials, including the Forester kangaroo (*Macropus giganteus*) (Johnson *et al.*, 1989) and the Tammar wallaby (*Macropus eugenii*) (Lynch *et al.*, 1993).

Both assays have been utilised on free-ranging *P. gunnii* known to have died of toxoplasmosis (Obendorf *et al.*, 1996). Specific cutoffs for MAT and DAT established for this species were used in this investigation. In confirmed cases of toxoplasmosis by Obendorf *et al.,* (1996), all except one *P. gunnii* had IgG-related seroconversions and one individual had a mixed IgM and IgG seroconversion. Where toxoplasmosis was considered to be the principle cause of death Obendorf *et al.,* (1996) confirmed only a few necropsy cases with identical DAT and MAT reactions of high to very high titres (up to 1:64000).

The severity of gross and histopathological findings of *P. gunnii* fed infected earthworms indicates that the experimental animals died as a result of acute generalised toxoplasmosis. These animals died several days earlier than those orally inoculated, at 11 and 14 DPI. The number of oocyst received by each *P. gunnii* in these cases is not known, but the pathogenicity of this strain at a dose of 100 oocysts given orally to *P. gunnii* had already been demonstrated. The short time intervals from exposure to death would first suggest that these animals ingested a larger infective challenge than orally inoculated *P. gunnii*, and as a consequence limited the detection of serum antibodies to *T. gondii.* The histopathological observations

suggest the rapid onset of fatal infection preceeded the formation of any detectible antibodies to *T. gondii.*

The route of infection has been shown to be significant in determining the variations of clinical illness. Experimental inoculation of various species such as mice, voles and sheep have indicated that infection is more certain, more rapid, and more likely to result in clinical illness after parenteral rather than oral inoculation (Findlay and Middleton, 1934; Olafson and Monolux, 1942; Jacobs and Hartley, 1964; Dubey and Frenkel, 1973; Frenkel, 1973). Intravenous or intraperitoneal inoculation appears more sensitive than the subcutaneous route, with intracerebral inoculation possibly the most sensitive route. There are reports of considerable variation in the virulence of naturally occurring strains of *T. gondii* (Frenkel, 1973; Beverley, 1976). Some of low virulence may increase in virulence after repeated passage through mice (Frenkel, 1973).

It is possible that because the earthworms were fed to *P. gunnii* during meal time, gastric juices and absorption mechanisms facilitated a more rapid break down of the oocyst wall and release of sporocysts in the small intestine. There was also a possibility that the selected individual animals were less immunologically competent than the other *P. gunnii* selected, but results of lymphocyte proliferative responses of these animals suggests this was not the case. Results are presented and discussed further in Chapter 8.

During the course of the disease, all *P. gunnii* displayed abnormal behaviour a few days prior to death. Diurnal movement and increased thirst was especially noticeable. No significant increase in body temperature was detected. Behavioural changes such as docility, apparent blindness, incoordination, atypical activity levels and loss in ability to seek refuge are often reported in free-ranging *P. gunnii*, detected with toxoplasmosis (Lenghaus *et al.*, 1990; Obendorf and Munday, 1990).

Due to the predilection of *T. gondii* for the central nervous system, the severe and extensive neurological damage with associated behavioural changes in *P. gunnii*, possibly increase their vulnerability to introduced predators and other hazards such as motor vehicles, through a loss of agility and a change from a noctural foraging behaviour to a diurnal habit (Lenghaus *et al.*, 1990; Obendorf and Munday, 1990). Studies on both wild and laboratory hybrid rats have confirmed that *T. gondii* can cause an increase in activity (Webster, 1994) and result in behavioural modifications that reduce the capacity of the infected animal to detect and avoid areas associated with high predation risk (Berdoy *et al.*, 2000). More importantly, it is possible that chronic parasitaemia of this nature further predispose *P. gunnii* to other diseases by decreasing their immune status, affecting fecundity and shortening their lifespan.

Normal behaviour of free-ranging *P. gunnii* may, however, predispose them to toxo-infection. For insectivorous species such as *P. gunnii,* the risk of browsing through soil infected with *T. gondii* for insects such as earthworms, is relatively high. *T. gondii* oocysts have been reported to remain infective in soil for up to 18 months (Frenkel *et al.*, 1975). Many millions of oocysts can be shed by a cat during the period of excretion in faeces (Dubey, 1998). It has been calculated that the number of oocysts shed in a 20 g cat stool can be in the order of 2 to 20 million oocysts and after faecal decomposition the local soil concentration can be a high as 100,000 oocysts / g (Frenkel *et al.*, 1975).

Earthworms passing through soil contaminated with decomposing cat faeces are capable of ingesting *T. gondii* oocysts (Frenkel *et al.*, 1975). Earthworms have an enormous capacity to affect microflora and faunal diversity of soil populations directly or indirectly (Brown, 1995). Their attraction to faecal material or soil enriched with faecal compost make earthworms important disseminators of some microbial pathogens due to their long ranging movements, and deposition of casts at different soil depths. Similarly, other soil-associated invertebrates which are

coprophagic are capable of acting as mechanical transport hosts for *T. gondii* oocysts through direct ingestion of cat faeces or soil contaminated with cat faeces (Markus, 1974; Frenkel *et al.*, 1975; Ruiz *et al.*, 1980). As domestic cats tend to bury their faeces in surface layers of soft soil, a range of invertebrates including ground beetles, isopods, molluscs and dipteran larvae may ingest infective oocysts of *T. gondii* by direct contact directly with feline faeces. Ingested oocyst can then be carried in their gut and dispersed through their discarded alimentary casts.

Earthworms (Annelida) and beetles (Coleoptera) make up a significant proportion of the diet of *P. gunnii* (Heinsohn, 1966; Quin, 1985; Brown, 1989; Dufty, 1994; Mallick *et al.*, 1997c). In a 3 year dietary study conducted by Mallick *et al.*, (1997c) the number of chaetae (microscopic body hairs of annelids) in faecal samples of free-ranging Tasmanian *P. gunnii* increased in the autumn and reached a peak in winter months. This corresponded to the seasonal peak in availability of earthworms.

The isolation and detection of *T. gondii* oocysts from earthworms feeding in oocyst seeded soil in the current study, support the potential role of earthworms as either transport or paratenic hosts for *T. gondii*.. The findings strongly suggest that earthworms can act as mechanical hosts of oocysts. The group of *P. gunnii* fed purged earthworms exhibited no clinical nor serological evidence of infection suggesting that infective stages do not establish in the somatic tissues of the earthworms and that such worms lose their infectivity by excreting the oocysts in their intestinal casts. For this reason earthworms are not considered long-term carriers of infective stages of *T. gondii.*

The proximity of earthworms to the surface layers of the soil makes them a readily available food source for birds or mammals and other intermediate hosts. *T. gondii* oocysts on grasses and other edible groundcovers are a recognisable source of infection (Dubey, 1994). Beyer *et al.*, (1994), confirmed that many wildlife species

ingest soil while feeding and grazing, acting as a means of exposure to viable oocysts. This is true for other animals such as sheep, where grazing is the principle form of *T. gondii* infection (Munday, 1970). These observations reinforce the threat faced by both herbivorous and omnivorous marsupials

Boorman *et al.*, (1977) emphasised such forms of transmission need to be considered prior to implementation of conservation, or rare species breeding programs, as mortalities can occur due to toxoplasmosis acquired in captivity or exacerbated in confined areas. Captive animals may often experience high stress levels resulting from confinement or change of habitat, which exacerbate latent infections with *T. gondii*. For these animals the risk of infection with toxoplasmosis can be minimised by controlling access of domestic or feral cats to feed and enclosures (Dobos-Kovacz *et al.*, 1974; Dubey *et al.*, 1988b)

The problem is obviously difficult to control for animals in a wild setting. Measures are required to minimise exposure of cats to the natural environment of *P. gunnii*. Numerous reports have shown very clearly the correlation between the presence of cats and the presence of the parasite in the area. An early study of the prevalence of antibodies to *T. gondii* in quokkas on Rottnest Island found that all individuals positive for *T. gondii* by serology and muscle biopsies, came from sites on the island, close to human settlements with domestic cats (Gibb *et al.*, 1966). A study on the sheep from a group of islands in the Bass Straits found less than 1% of sheep bred on cat-free islands to be seropositive, as opposed to almost 30% of sheep being seropositive on cat-infested islands (Munday, 1972b). The encroachment of humans and their pets to natural forests of Tasmania is growing. Rural and urban areas are continually expanding and the number of feral cats are increasing at an alarming rate. This interaction places *P. gunnii* at greater risk of disease as the impact of habitat changes and predation places extra pressure on their survival.

Chapter 4. *Hepatozoon* sp., and other blood parasites *P. gunnii*

4.1 INTRODUCTION

It has become clear that *Toxoplasma gondii* is a major pathogen of *Perameles gunnii*, but there are few descriptions of blood parasites or other diseases of these animals (Obendorf and Munday, 1990). Recent parasitological work on this temperate marsupial has revealed what appears to be the first record of an *Hepatozoon* species from a Tasmanian bandicoot, including the first report of a trypanosome species.

The original description of an *Hepatozoon* species in bandicoots was by Welsh and Dalyell (1909). The examination of a peripheral blood slide of a long-nosed bandicoot (*Perameles nasuta*) from New South Wales revealed a number of extracellular elongated, cylindriform parasites of regular outline which they took to be haemogregarines, describing them as *Haemogregarina peramelis*, Family Haemogregarinidae (Welsh and Dalyell, 1910). In 1926, Wenyon placed all the haemogregarines of mammals into the genus *Hepatozoon* and this species became known as *Hepatozoon perameles* (Mackerras, 1959a). The reclassification among species found in marsupials was based on the identification of gametocytes infecting either erythrocytes or leukocytes. The species was further redescribed, and extracellular as well as intracellular gametocytes were documented in the short-nosed bandicoot (*Isoodon obesulus*) from Innisfail in northern Queensland (Mackerras, 1959a). Mackerras (1959a) found the infections were less common in animals from the southern Queensland areas of Gympie and Brisbane. The most recent report of a *Hepatozoon* species in a bandicoot was by Anderson (1990) who detected the parasites in thin blood smears of the New Guinea Spiny Bandicoot, *Echymipera kalubu*. Intraerythrocytic gametocytes were demonstrated in 41% of animals captured. No *Hepatozoon* species have been reported from the temperate regions of Australia.

Many *Hepatozoon* infections have been reported amongst mammals but only a few investigations have closely analysed the pathogenic effects of the species. While a few *Hepatozoon* infections are reported to have harmful effects on their hosts such as *H. muris* and *H. balfouri* in the rodent (Furman, 1966, Clark *et al.*, 1973) and *H. canis* causing fever, anaemia and fatal infections in some dogs (Panciera *et al.*, 1997; Macintire *et al.*, 1997), not all reports of hepatozoonosis in mammals suggest they are pathogenic. In order to further clarify clinicopathological characteristics of hepatozoonosis in *P. gunnii*, observations were made on naturally infected animals held in captivity over an extended period of time and the progress of parasitaemia on a splenectomized animal and following treatment with corticosteriods. It is generally considered that, as splenetomized animals are naturally susceptible to fulminant protozoan infections (Garnham, 1963), *Hepatozoon* species might potentially cause acute clinical disease following the removal of the spleen in *P. gunnii*.

Despite intensive studies on the ultrastructure of certain members of the Apicomplexa, little information is available on the ultrastructure of *Hepatozoon* spp., in vertebrates, except for miscellaneous studies on stages in dogs, snakes, frogs, and mosquitos (Vivier *et al.*, 1972, Bashtar *et al.*, 1984, Droleskey *et al.*, 1993, Lowichik *et al.*, 1993, Desser *et al.*, 1995). What is known is that ultrastructural characteristics shared by haemogregarines and other eimeriid coccidia include the presence of rhoptries, polar ring, microtubules, micronemes, conoid and trilaminal pellicle with some variations occuring in the number and position of rhoptries, pellicle structure and zoite dimensions (Bashtar *et al.* 1984). Transmission electron microscopy was performed on *Hepatozoon* from *P. gunnii*.

4.2 MATERIALS and METHODS

4.2.1 Animals

In the present study 220 *P. gunnii* were live trapped at two sites in the Huon Valley region of southern Tasmania in accordance with established guidelines and protocols of Parks and Wildlife Service permit to take protected wildlife (Hobart, Tasmania, Australia; Permit numbers 94011, 95249, 95256, 96197). Approval was received from the University of Tasmania Ethics Committee (Animal Experimentation; Investigations No. 95082 and 96081). Blood samples were collected from the peripheral ear vein immediately after the animal was restrained as described in methods of blood collection, Chapter 2; 2.2. Thin blood smears were prepared in the field or upon return to the laboratory. The splenectomies were performed by Dr Barry Wells at the Kingston Veterinary Hospital and all animals were housed at the Central Animal House at the University of Tasmania.

4.2.2 Staining of Blood Smears (Chapter 2; 2.2)

The most commonly used stains for blood parasites are the Romanowsky stains. Several other stains can be used for the detection of blood parasites but these may not allow differentiation between various cells and highlight subtle distinctions between shades of dye up-take by cellular material.

Thin smears made from trapped animals were air dried, fixed with absolute methanol, stained with Leishman's (Media Makers, Australia) or freshly made Leishman's and examined under oil immersion lens (x100). Duplicate smears were made using Giemsa (Gibco Diagnostics, USA) 1: 20 with Buffered PBS (pH 6.8). A 50:50 mixture of Leishman's / Wright's stain was often used for comparative value and performed using an Ames Hema-Tek Slide Stainer (England) at the Department of Haematology of the Royal Hobart Hospital (RHH). When applying the Leishman's / Wright's stain the preferred choice was the conventional manner as described by Mitruka and Rawnsley, (1977) rather than the dip technique.

4.2.3 Haematological Data Analysis

Haematological values were obtained for *Hepatozoon* spp., infected and uninfected *P. gunnii*. Data was obtained using an automated cell counter system (Technicon H2 Automated Counter, Dublin, Ireland) and manual white cell counts as described in Chapter 2; 2.2.

4.2.4 Detection of Parasites

The presence of *Hepatozoon* gametocytes in the peripheral blood under light microscopy using oil immersion lens (x100) served as the basis for identifying an infection with the parasite. Detection was based upon visual confirmation of *Hepatozoon* gametocytes. Known positive smears and light photomicrographs were used as controls. Detection of a single gametocyte was considered positive for that animal while no sightings were recorded "no parasite seen (NPS)". An additional screening technique was that developed to concentrate parasites. This technique was recommended by Cavill and Goldsmid, (1972) when parasitaemia was considered low. Heparinised capillary tubes were three-quarters filled with blood, sealed and centrifuged for ten minutes in a microhaematocrit centrifuge (Hawksley, England). The buffy coat section was examined under light microscopy for the presence of other blood parasites.

4.2.5 Detection of Parasites using Acridine Orange O (Chapter 2; 2.2.7)

Thin and thick blood slides were stained with the fluorochrome Acridine Orange O as described by Cranston and Goldsmid, (1972). The blood slides were fixed in methanol and stained for 3 seconds in Acridine Orange O diluted in 0.01 per cent Sorensen's Buffer, pH 5.4. The films were then washed in two changes of buffer and finally in distilled water. The slides were then air-dried and examined under an ultra-violet microscope.

4.2.6 Measuring of Parasite Dimension Using the Image Analyser

Measurements were made on parasites from collected slides and were included with additional data performed by Lê, (1996). Lê, (1996) extended the study to include the Southern Brown Bandicoot (*Isoodon obesulus*) and other Tasmanian marsupials. Through this joint effort, the dimensions of 100 parasites from *P. gunnii* were recorded utilising image analysing equipment described in Chapter 2; 2.2.10.

4.2.7 Cell Culture

Larger volumes of blood (6 mL) were taken from parasite positive animals and processed using peripheral blood mononuclear separation techniques described in Chapter 2; 2.8. A Ficoll-gradient was used to concentrate white blood cells and parasites. Anticoagulated venous blood was diluted 2:1 in RPMI 1640 medium (Commonwealth Serum Laboratories, Victoria, Australia) and layered onto 12ml of Histopaque-1077, a solution of polysucrose 5.7g/dL and sodium diatrizoate 9.0 g/dL with a density of 1.077=0.0001g/ml (Sigma NSW, Australia). The erythrocytes and granulocytes aggregated and rapidly sediment, while the lymphocytes, other mononuclear cells and parasites remained at the plasma - Histopaque interface. This layer was suctioned off and the parasites and cells were kept in a cell culture medium at 37°C in 5% carbon dioxide for 5 days. The cell culture medium included RPMI 1640 and foetal calf serum (FCS) 10% (v/v) (CSL, Victoria, Australia), L-glutamine 2 mmol 5% (v/v) (ICN Flow Laboratories, NSW, Australia), benzylpenicillin 100U/ml (CSL, Victoria, Australia), 100 µg/ml streptomycin (CSL, Victoria, Australia). Samples were fixed and stained for light microscopy. Samples were processed for transmission electron microscopy (TEM) as described Chapter 2; 2.3.

4.2.8 Screening Captive Animals Naturally Infected with *Hepatozoon* spp.

Two animals naturally infected with *Hepatozoon* spp., and two uninfected animals were kept in captivity at the Central Animal House at the University of Tasmania

over a 15 month period. Blood smears were made at weekly intervals and parasite counts performed. Blood samples were treated and processed for transmission electron microscopy and cell concentration techniques. Control animals were held for a period of 2 to 3 months for a comparison of haematological values.

4.2.9 Splenectomy

To investigate the role of the spleen in *Hepatozoon* infected animals and parasitaemia levels, survival surgery with splenectomy was performed on a free-ranging and naturally infected bandicoot. Surgery was performed by a qualified Veterinary surgeon, Dr Barrie Wells of the Kingston Veterinarian Hospital, in southern Tasmania. A pre-splenectomy screen of a peripheral blood smear was made to assess parasitaemia levels. Animals were serologically tested to exclude toxoplasmosis, Chapter 2; 2. Following surgery the animal and its health status was observed and the intensity of parasitaemia evaluated. A blood sample was taken "post-splenectomy (day 0)". Blood smears were taken daily after the recovery period of 3 days post splenectomy to assess parasite levels. The removed spleen was measured and processed by routine histology Chapter 2; 2.4, and screened at length for the presence of schizonts.

On day 25, splenectomised animals received 0.05 mg / mL intramuscular injection of a synthetic cortisone derivative with a composition of Betamethasone (Betsolan, Jurox Pty Ltd, Rutherford, NSW), once a week for four weeks. The animal was observed for 4 weeks and then euthanased by barbituate overdose. Liver, spleen, kidney, heart, lung and brain tissues were removed at necropsy and fixed in 10% buffered formalin and sections prepared by routine histology. Imprints were made from the above organs and stained in the same manner as the blood films using Giemsa stain.

4.2.10 Concentration Technique

If parasitaemia was evaluated as low, a concentration technique was deemed to be necessary for the purposes of Transmission Electron Microscopy (TEM). A variation of the microhaematocrit technique Goldsmid, (1970) and Cavill and Goldsmid, (1972) was applied.

Heparinised capillary tubes were filled to three-quarters of their capacity with blood collected from *P. gunnii.* The tubes were sealed with sealer and spun for 10 minutes in a Hawksley microhaematocrit centrifuge (Hawksley, England). Immersion oil was placed across the buffy coat section of the tubes that were examined under light microscopy at low power (x10 magnification) for the presence of extracellular motile blood parasites. Capillary tubes for studies involving the *Hepatozoon* parasite were processed for TEM.

4.2.11 Transmission Electron Microscopy (Chapter 2; 2.3).

Positive samples with *Hepatozoon* sp., were processed for transmission electron microscopy (TEM) using the concentration technique Cavill and Goldsmid, (1972). Heparinised capillary tubes were centrifuged and the buffy section examined under light microscopy for the presence of other blood parasites. The portion of the capillary tube containing the buffy coat was excised using a diamond knife and then immersed into 2.5% glutaraldehyde (Ajax Chemicals, Sydney, Australia) in 0.2% cacodylate buffer (BDH Chemicals Ltd, Poole, England). The tube segments were left overnight until the cellular plugs were solid enough to be removed by gentle force. The plugs were stored in cacodylate buffer until processing for TEM.

Buffy coat plugs post fixed with 1% osmium tetroxide and 4% uranyl acetate (BDH Chemicals Ltd, Poole, England) and then dehydrated in ascending concentrations of alcohol. Propylene oxide (Merck Pty Ltd, Kilsyth, Victoria) was used as a

transitional solvent and the specimens were infiltrated and embedded in Epon resin in polyethylene Beem capsules (Alltech, Australia, Pty. Ltd.). The resin blocks were then polymerised at 60°C for 48 hours.

Semi-thin survey sections (0.5 μM) were cut using a Reichart Ultracut S microtome (Leich AG, Austria) and stained using toluidine blue (BDH Chemicals Ltd, Poole, England). Ultra-thin sections (70 nm) were then cut, dried and stained with 1% aqueous uranyl acetate for 30 min and post-stained with lead citrate. Examination of thin sections was performed at the Department of Pathology using a transmission electron microscope (EM 410LSE, Phillips, Holland).

4.2.12 Scanning Electron Microscopy (Chapter 2; 2.3)

SEM was used to visualise any structural deformation in erythrocytes infected with *Hepatozoon* sp., or changes to its membrane. Blood was collected from infected *P. gunnii* into 1.5 mL collecting tubes filled with 0.75 mL with Alsever's solution Chapter 2; 2.3, to preserve the sample and prevent coagulation. Lê, (1996) trialed several processing techniques described by Merchant and Thomas (1983) to ascertain which produced the optimal results. The best results required a 50:50 ratio of blood : Alsever's solution and the solution mixed gently by inversion.

4.3 RESULTS

4.3.1 Detection of Parasites and Measurements

Fifty-five of the 220 adult *P. gunnii* (25%) examined were found to be infected with parasites which were present as both extracellular and intracellular gametocytes. Screening of blood slides determined the collective parasite counts for *P. gunnii*. Lê, (1996) confirmed the highest parasitaemia observed on *P. gunnii* were 2 parasites per 1000 erythrocytes.

Using image analysis, the size of the *Hepatozoon* parasites were found to range from 8.5 to 9.5 μM in length, by 2 to 3 μM in width. The nucleus stained bright red with infected erythrocytes being prominently enlarged and oval. Intracellular forms were often seen on the blood film. Infected cells were enlarged, oval and usually about 8 x 10 μM in size (Figure 4.1). Occasionally two gametocytes were present in one red blood cell (Figure 4.2). Often gametocytes appeared enlarged in all diameters, measuring 12 μM by 10 μM; in these cases, the nucleus was enlarged and the chromatin stained throughout (Figure 4.3). With Leishman's stain the chromatin of the nucleus was coloured bright red or reddish-purple, and was distributed in the form of a strongly marked, limiting membrane or as one or more irregularly-rounded masses on or near this membrane. The cytoplasm stained a faint and uniform blue, and offered a marked contrast to the strongly staining chromatin of the nucleus.

Extracellular forms were often identified in the blood films of *P. gunnii* (Figure 4.4). Acridine Orange O trialed for detection of parasites, was observed to be a suitable alternative or complimentary stain for screening blood slides for *Hepatozoon* gametocytes with UV microscopy. Figure 4.5, highlights the gametocyte in a blood film from a marsupial with low parasitaemia.

During the collaborative investigations with Lê, of parasites in *P. gunnii*, trypanosomes were detected (Figure 4.6). Lê, (1996) initially observed the parasite in peripheral blood smears of a Southern Brown Bandicoot, *Isoodon obesulus* (Figure. 4.7) and re-examination of blood smears of *P. gunnii* revealed trypanosomes in peripheral circulation of this species too.

4.3.2 Transmission Electron Microscopy (TEM)

Blood samples known to be infected with *Hepatozoon* were processed for electron microscopy by first concentrating parasite numbers using the microhaematocrit technique described in Materials and Methods. Concentration of the parasite were

Figure 4.1

Blood film from *P. gunnii* shows erythrocyte infected with *Hepatozoon* gametocyte. Gametocyte has a well stained nucleus and internal structures. Note the nucleated erythrocyte.

Leishman's stain.
Bar = 10 μM

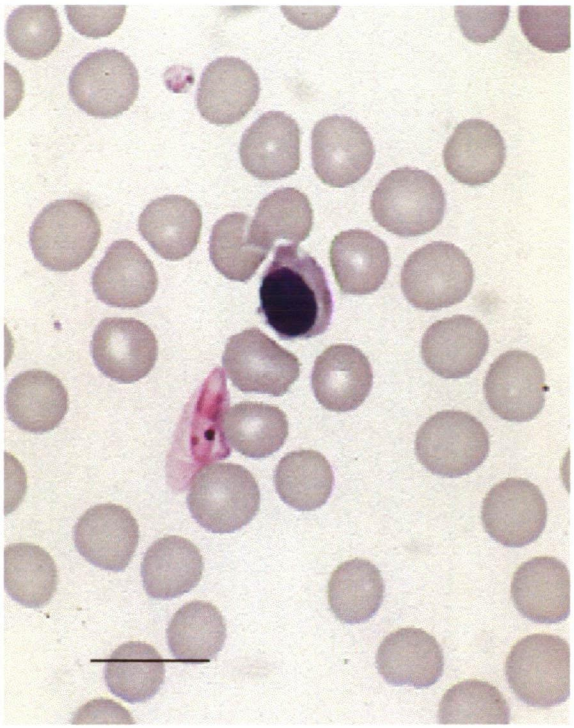
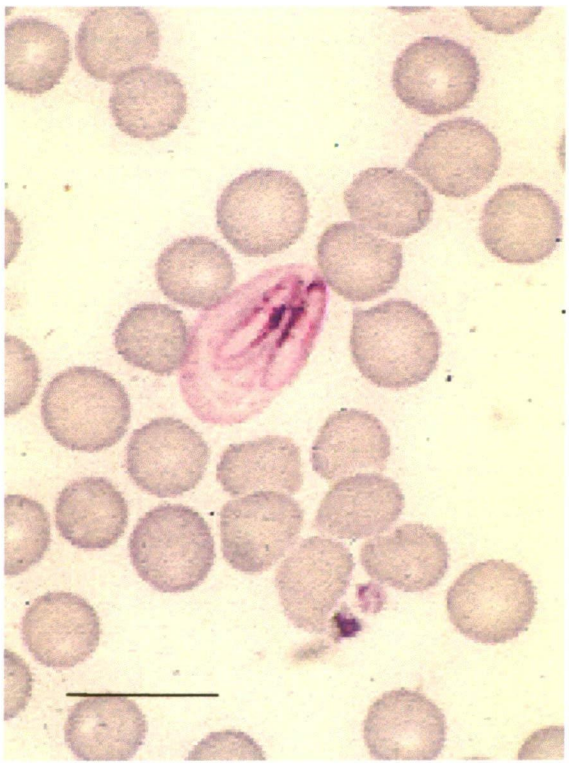


Figure 4.2

Blood film from *P. gunnii* shows two intracellular *Hepatozoon* gametocytes in erythrocyte. Host cell is grossly enlarged.

Leishman's / Wright's stain.
Bar = 10 μM



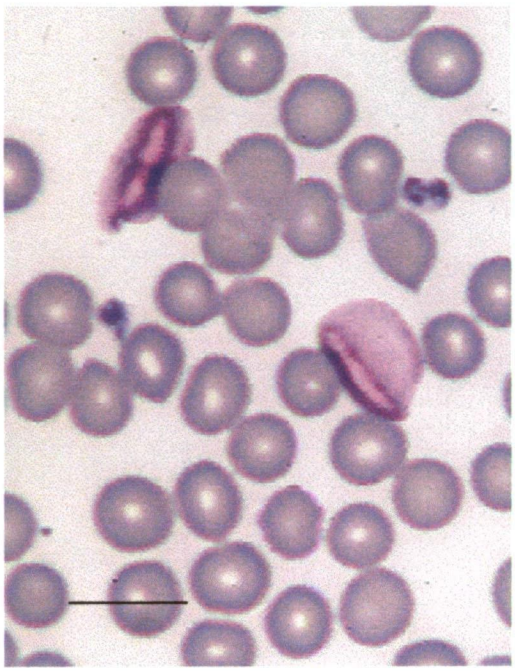


Figure 4.3

Blood film from *P. gunnii* shows enlarged gametocytes measuring 12 μm by 10 μm. The nucleus is also enlarged and the chromatin is stained throughout.

Leishman's stain.
Bar = 10 μM

Figure 4.4

Extracellular *Hepatozoon* gametocyte seen in a blood film from *P. gunnii.*

Leishman's stain.
Bar = 10 μM

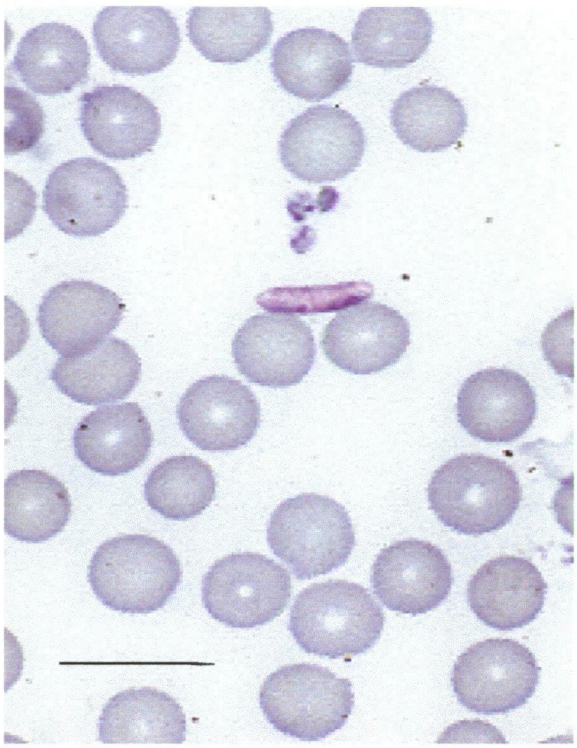


Figure 4.5

Hepatozoon gametocyte stained with Acridine Orange O and detected using UV microscopy.

Acridine Orange O stain.
Bar = 10 μM

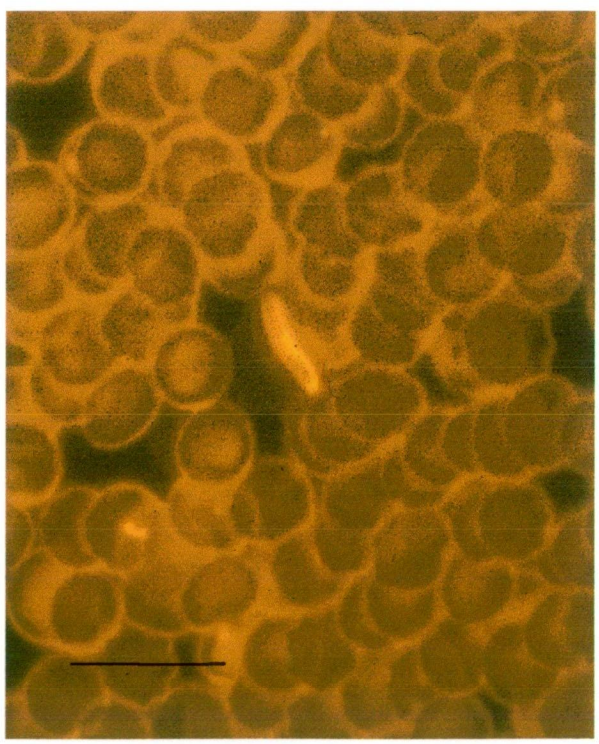


Figure 4.6

Light microscopy of a trypanosome from the peripheral blood smear of *P. gunnii.*

Leishman's stain.
Bar = 10 μM

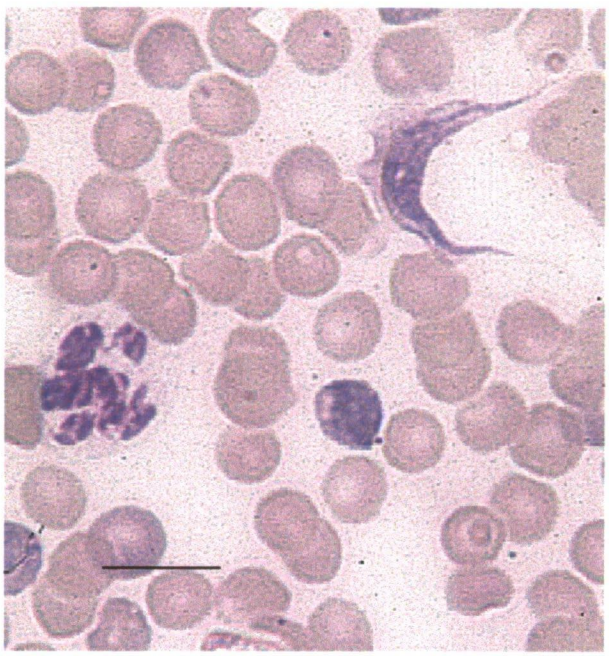
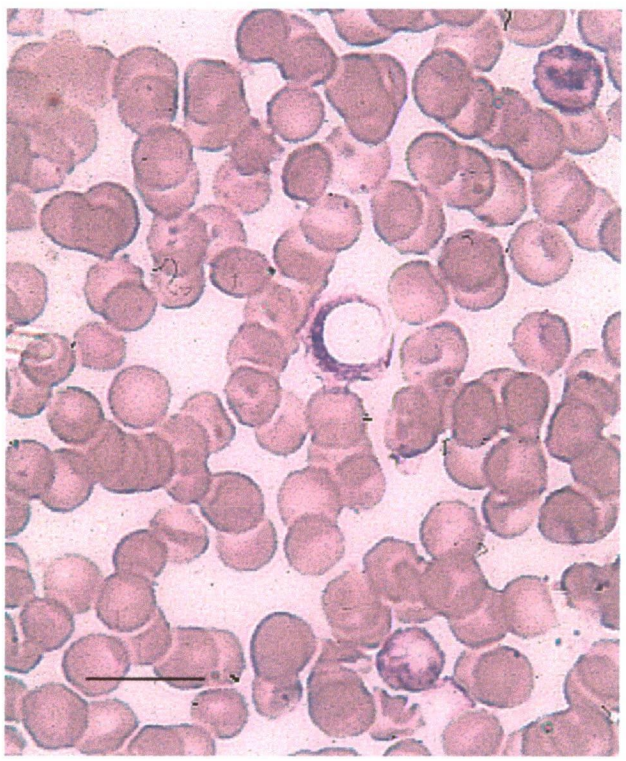


Figure 4.7

Trypanosome from the peripheral blood smear of the Southern Brown Bandicoot, (*Isodoon obesulus).*

Leishman's stain.
Bar = 10 μM



best seen below the erythrocyte-buffy coat interface. Intracellular forms of *Hepatozoon* gametocytes were found within erythrocytes under the transmission electron microscope. Parasites were electron dense and easily distinguished from uninfected cells in which there were no parasites. Parasites appeared as ovoid intracellular bodies when transversely sectioned (Figure 4.8). Evidence of intracellular organelles such as Golgi apparatus within parasitised erythrocytes were observed. The cytoplasm of all erythrocytes presented with a granular consistency in which patches of electron pale areas occurred throughout the cell (Figure 4.9). On occasion hair-like processes were observed extending from the plasmalemma of some infected erythrocytes. The conoid was identified between the polar ring at the apical pole of the parasite (Figure 4.10a). The subpellicular microtubule array was evidenced by its truncation two-thirds the way down the cell. It was clearly recognised by a dual-layered membrane consisting of two opposed identical layers (Figure 4.10b). Micropores were not observed. Rhoptries are not shown here, but Lê, (1996) described rhoptries protruding into the cytoplasm from the anterior end and mitochondria, with tubular cristae situated centrally and anterior to the nucleus. The most prominent cytoplasmic structure was the abundance of micronemes within all gametocytes and varied in morphology from round to pyriform, depending on the angle of section.

4.3.3 Scanning Electron Microscopy (SEM)

The polylysine coated coverslip technique proved adequate in the analysis of parasitised blood samples. Blood cell preparation from an infected bandicoot revealed erythrocytes with gross cell surface modifications (Figure 4.11). The erythrocytes were distinctly different from the majority of erythrocytes that displayed smooth cell surfaces. Membrane deformations occurred primarily along the longitudinal axis of the erythrocyte.

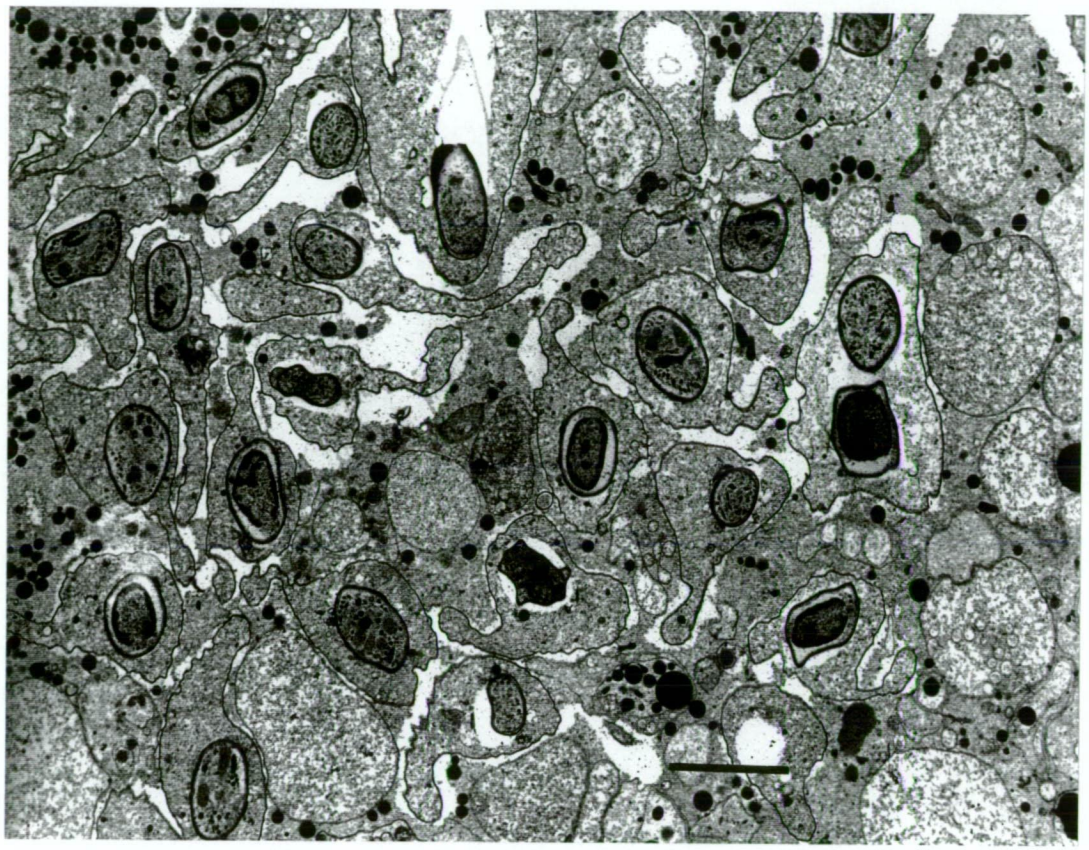


Figure 4.8 Transmission electron micrograph of concentrated parasites in packed erythrocyte layer. Parasites appear as ovoid intracellular bodies.

Magnification: X 6000

Bar = 5 μm

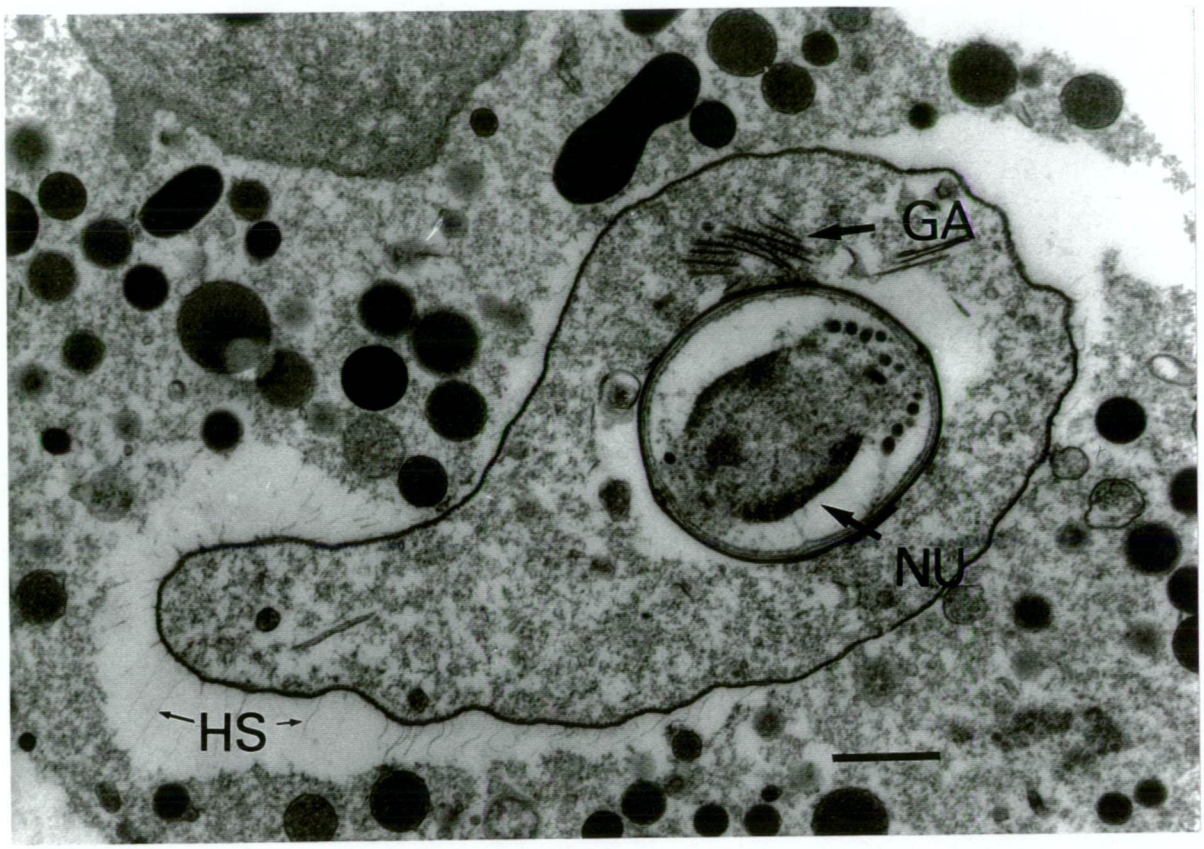


Figure 4.9. Electron micrograph of host erythrocyte with intracellular *Hepatozoon* gamont (transverse section). Host cell possess organelle remnants with hair-like projections from the membrane.

GA = Golgi apparatus, HS = Hair-like structures, NU = Nucleus

Magnification: X 28, 000
Bar = 1 μm

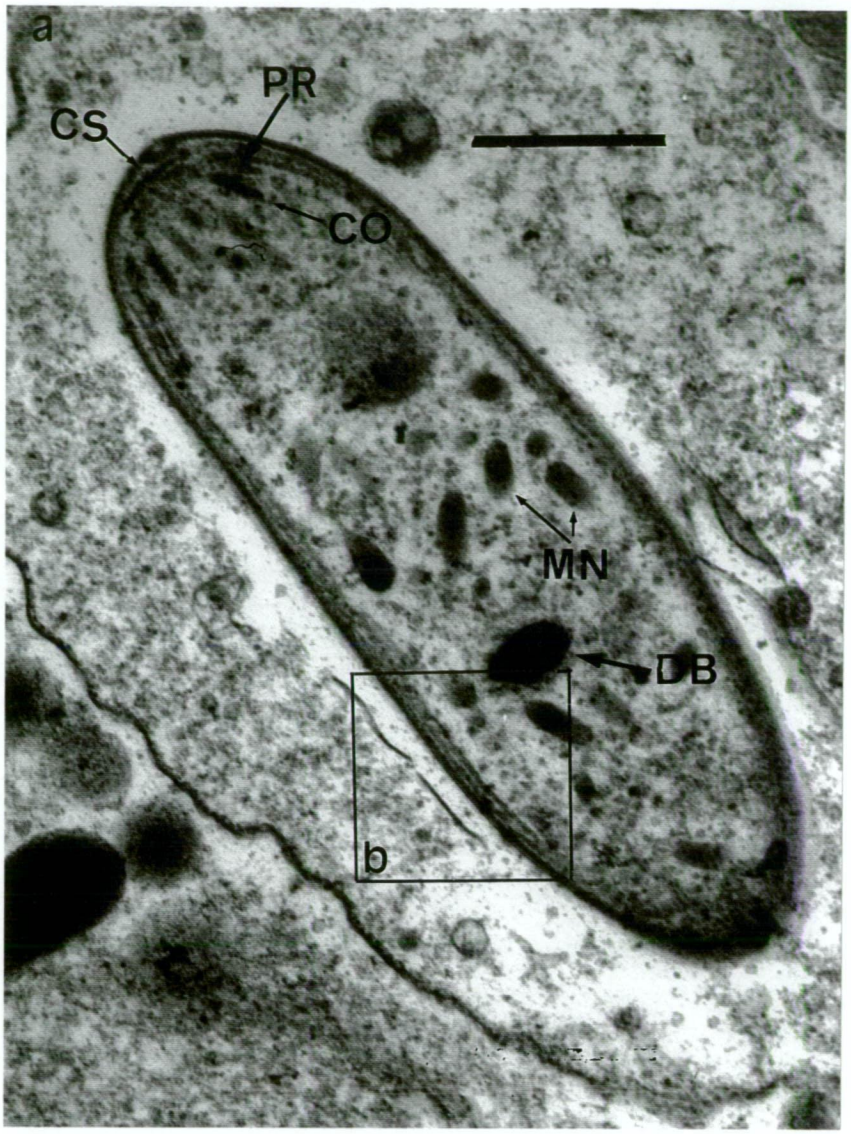


Figure 4.10 **(a)** Electron micrograph of *Hepatozoon* gamont (longitudinal section). Apical pole features the polar rings and conoid.

PR = Polar ring, DB = Electron dense body, MN = Micronemes, CO = Conoid, **(b) inset** SM = Subpellicular microtubule.

Magnification: X 28, 000

Bar = 1 μm

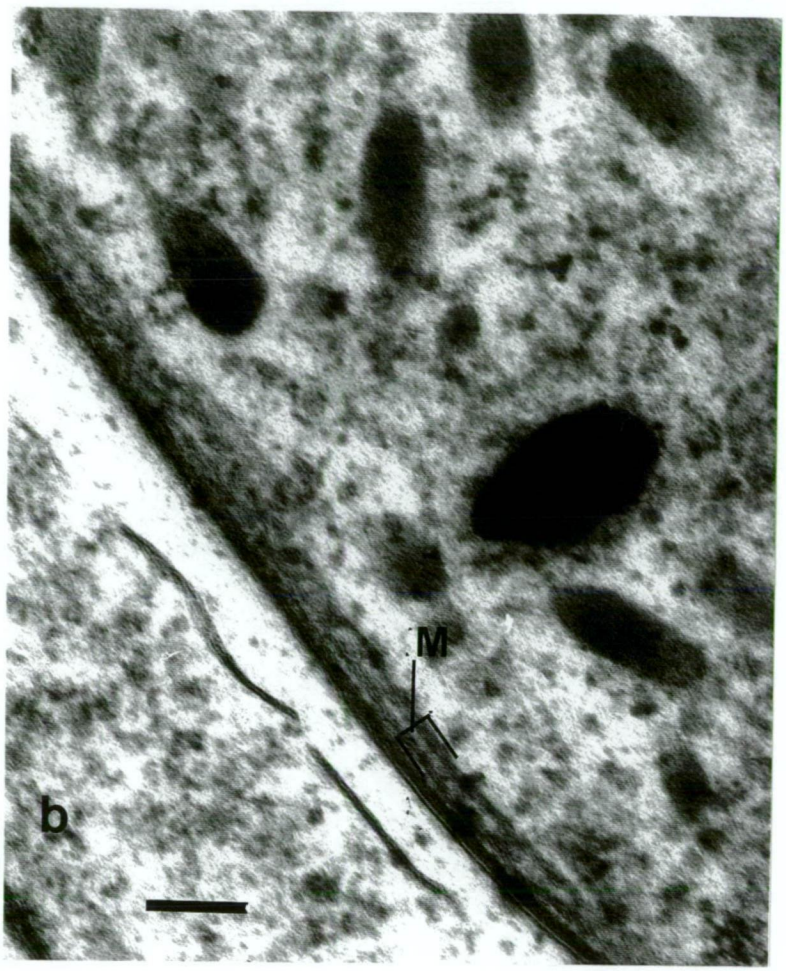


Figure 4.10 (b). Subpellicular microtubule array extending two-thirds the way down the cell. The tubule is possess a high electron density membrane.

Magnification: X 126,000

Bar = 0.1 μm

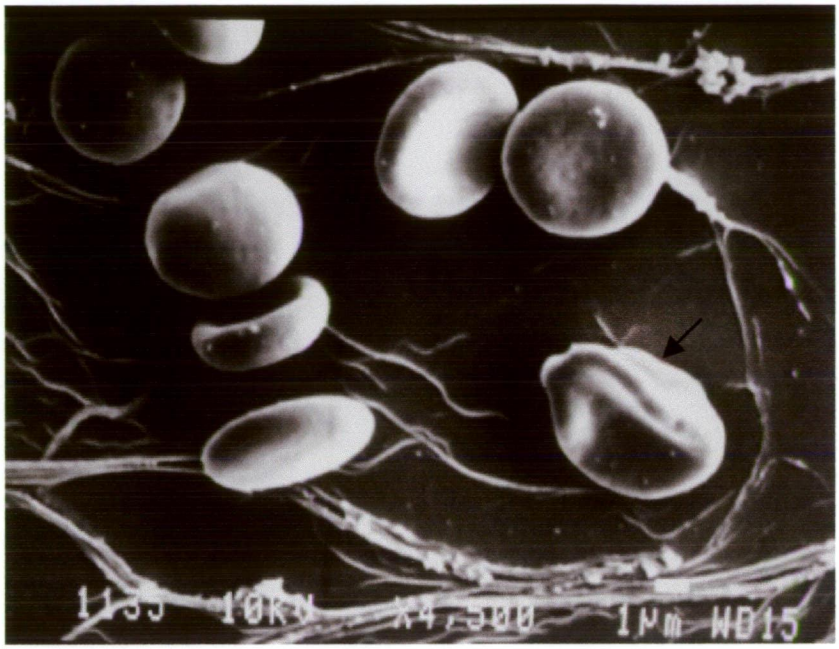


Figure 4.11. Scanning electron micrograph of normal erythrocytes surrounding a cell with membrane distortion consistent with *Hepatozoon* shape and proportions (arrow).

Bar = 1 μM

4.3.4 Haematological Analysis

Descriptive statistics of *P. gunnii* haematological data collected from animals, including non-parasite infected and parasite-infected ones, are presented in Table 4.1. Data was analysed using Mann-Whitney U-test and Student's t-test assuming unequal variance and presented in Table 4.2. The results revealed that difference in blood parameters between non-parasite and parasite-infected animals could be reasonably attributed to chance. A comparison of males and females between non-parasite and parasite-infected groups was made (Table 4.3). There is evidence of a difference in MPV (mean platelet volume) between males and females ($p = 0.018$) of these groups. However, all other differences seen between males and females in all other blood parameters could be reasonably attributed to chance. Raw data is presented in Appendix 11.4a.

A comparison of males and females with the blood parasite, *Hepatozoon* sp., revealed any differences in blood parameters between the two groups could be reasonably attributed to chance. Descriptive statistics of *P. gunnii* haematological data collected from parasite-infected bandicoots only, are presented in Table 4.4. Data was analysed using Mann-Whitney U-test and Student's t-test assuming unequal variance and presented in Table 4.5. All raw data is presented in Appendix 11.4b.

4.3.5 Cell Culture

The preliminary investigation of concentrating parasites using Ficoll-gradient was successful. A significant number of parasites were collected from an animal with low parasiteaemia. The media provided a good source of maintenance for the parasite for approximately 5 days incubation. Gametocytes can be seen in culture (Figure 4.12) and stained with Leishman's with evidence of good morphological features (Figure 4.13).

	No	Mean	Median	Maximum	Minimum	25th Percentile	75th Percentile
WBC x10^9/L	15	4.77	3.70	9.78	1.90	2.42	8.37
RBC x10^{12}/L	30	7.75	7.74	9.62	6.21	7.01	8.38
HGB (g/L)	30	142.57	141.50	177.00	115.00	133.00	150.00
HCT (L/L)	30	0.41	0.41	0.52	0.28	0.38	0.43
MCV (fL)	30	52.14	52.30	57.70	45.80	51.00	53.70
MCH (pg)	30	18.26	18.15	21.80	15.60	17.80	18.70
MCHC (g/L)	30	348.20	345.00	394.00	325.00	336.00	356.00
RDW (%)	30	14.55	14.50	17.10	13.20	13.90	15.10
HDW (g/L)	30	24.42	23.95	33.40	20.60	22.70	25.40
PLT x10^9/L	30	119.43	106.50	326.00	22.00	65.00	138.00
MPV (fL)	30	4.61	4.30	8.50	2.70	3.60	5.50
PDW (%)	29	80.84	82.20	100.60	56.20	71.50	90.60
PCT (L/L)	28	0.017	0.002	0.080	0.000	0.001	0.021

Table 4.1 Descriptive Statistics, including all data (i.e., both non-parasite and parasite (*Hepatozoon*) infected animals).

Data was analysed using Mann-Whitney U-test	
	p-value
WBC	0.814
RDW	0.064
PCT	0.208
Data analysed using a t-test assuming unequal variance	
	p-value
RBC	0.922
HGB	0.724
HCT	0.445
MCV	0.713
MCH	0.413
MCHC	0.721
LOGHDW	0.302
LOGPLT	0.215
LOGMPV	0.794
PDW	0.723

Table 4.2 Comparison of non-parasite and parasite (*Heptazoon*) infected animals.

Data was analysed using Mann-Whitney U-test	
	p-value
WBC	0.540
RDW	0.115
PCT	0.905

Data analysed using a t-test assuming unequal variance	
	p-value
RBC	0.681
HGB	0.505
HCT	0.424
MCV	0.559
MCH	0.768
MCHC	0.096
LOGHDW	0.147
LOGPLT	0.882
LOGMPV	0.018
PDW	0.120

Table 4.3 Comparison of males and females between non-parasite and parasite (*Hepatozoon*) infected animals.

	No.	Mean	Median	Maximum	Minimum	25th Percentile	75th Percentile
WBC x10^9/L	6	4.33	3.24	8.37	2.42	2.60	6.09
RBC x10^{12}/L	11	7.73	7.78	8.92	6.52	6.92	8.52
HGB (g/L)	11	143.73	139.00	169.00	128.00	134.00	152.00
HCT (L/L)	11	0.42	0.41	0.52	0.37	0.39	0.42
MCV (fL)	11	51.87	53.10	57.70	45.80	49.60	54.20
MCH (pg)	11	17.98	17.80	20.20	15.60	16.70	18.70
MCHC (g/L)	11	346.82	345.00	372.00	325.00	336.00	358.00
RDW (%)	11	14.93	15.10	17.10	13.20	14.60	15.30
HDW (g/L)	11	25.31	24.10	33.40	20.60	22.70	26.70
PLT x10^9/L	11	124.64	122.00	263.00	60.00	101.00	138.00
MPV (fL)	11	4.67	4.40	7.90	3.00	3.60	5.50
PDW (%)	11	81.85	85.30	95.10	57.80	75.30	92.20
PCT (L/L)	11	0.022	0.010	0.080	0.001	0.001	0.040

Table 4.4. Descriptive Statistics, including parasite infected bandicoots only.

Data was analysed using Mann-Whitney U-test	
	p-value
WBC	0.064
RDW	0.854
PCT	0.709
Data analysed using a t-test assuming unequal variance	
	p-value
RBC	0.085
HGB	0.267
HCT	0.694
MCV	0.785
MCH	0.718
MCHC	0.281
LOGHDW	0.447
LOGPLT	0.559
LOGMPV	0.320
PDW	0.168

Table 4.5. Comparison of males and females with blood parasite, *Hepatozoon*.

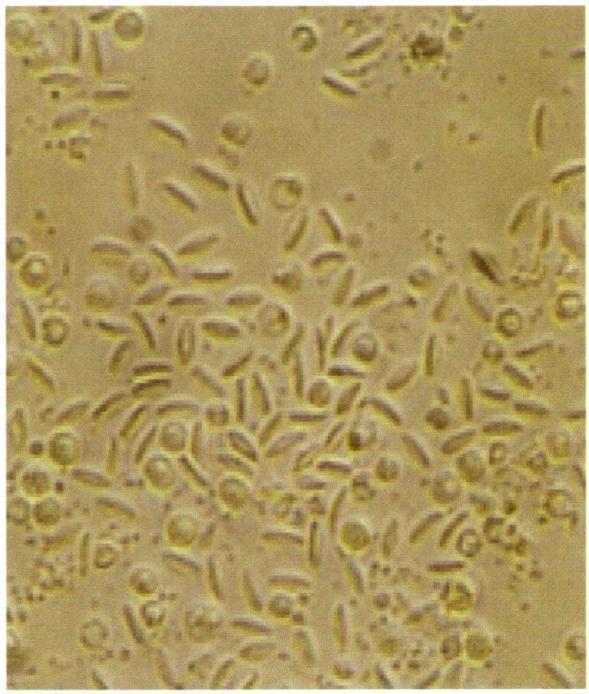


Figure 4.12

Hepatozoon gametocytes separated from whole blood of *P. gunnii* and maintained in cell culture. Note the round mononuclear cells in culture.

Leishman's stain
Magnification x40

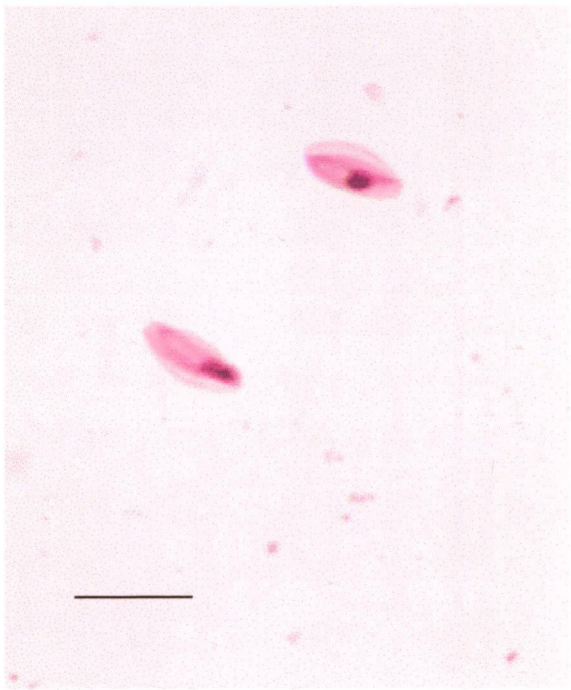


Figure 4.13

Hepatozoon gametocytes from cell culture on Day 4. Nucleus is well stained.

Leishman's stain.
Bar = 10 μM

4.3.6 Splenectomy and Cortisone Injections

The animal that underwent splenectomy showed no clinical signs of illness associated with the *Hepatozoon* infection. There was no evidence of weight loss or change in body temperature post-recovery. Peripheral blood samples taken from day 6 to the final sample at day 25 failed to reflect any increase in *Hepatozoon* gametocyte numbers. Full blood counts displayed a marked lymphocytosis in the first three days post-splenectomy, a feature reflected in the corresponding blood smear. White blood cell counts increased from 7.9 $x10^9$ / L to 11.0 $x10^9$ / L. Smears from day 3 showed a decrease in this initial rise and stabilised at 7.8 $x10^9$ / L. By day 25, the mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) values, and (PLT) platelet counts had decreased. See Table 4.6.

Blood smears were taken after the animal received weekly cortisone injections starting on day 25. No increase in *Hepatozoon* gametocyte number was observed during this period. At no stage was there an increase in parasitaemia

4.3.7 Captive Animals Naturally Infected with *Hepatozoon* spp.

No clinical signs of illness attributable to *Hepatozoon* infection were observed in captive bandicoots. All bandicoots with circulating gametocytes on initial examination had a decrease in number of gametocytes after 3 months of captivity. Animals held in captivity for 10 months showed no circulating gametocytes.

Parameter	Pre-splenectomy	Day 0	Day 25
WBC $x10^9$/L	7.90	11.04	7.88
RBC $x10^{12}$/L	7.64	7.49	7.86
HGB (g/L)	134	127	128
HCT (L/L)	0.42	0.39	0.40
MCV (fL)	54.1	51.7	52.1
MCH (pg)	19.2	17.0	16.4
MCHC (g/L)	352	328	314
RDW (%)	17.2	16.4	15.0
PLT $x10^9$/L	331	321	228
MPV (fL)	6.8	6.9	8.2
PDW (%)	53.2	54.7	48.7

Table 4.6 Results of blood count *P. gunnii* pre and post - splenectomised on Day 0 and Day 25.

4.4 DISCUSSION

The stages of *Hepatozoon* seen in the blood smears of *P. gunnii* were consistent with those of mature gametocytes described in the study of *Hepatozoon* by Mackerras (1959a). No other stages were observed in *P. gunnii* blood films. Though the appearance of post-merozoite forms (trophozoites) of this genus have been reported in peripheral blood of snakes it was considered to be a rare occurrence or "accidental factor" by Mackerras, 1962 (cited by Ball *et al.*, 1967). There appears to be no reliable criteria for differentiating between young gametocytes and trophozoites found in the peripheral blood (Ball *et al.*, 1967).

The gametocytes, both intracellular and extracellular forms, were uniform in appearance. Parasites that infected erythrocytes produced marked alterations in the gross morphology and quite often caused changes in the properties of the plasma membranes of the erythrocytes. A number of elongated bodies lying free in the plasma contained a nucleus but were devoid of pigment. Intracellular forms exclusively parasitised erythrocytes with the rare occurrence of invasion by two gametocytes. No merozoites were observed within the erythrocytes. Most of the parasites had a very regular outline and definite structure, and were almost of uniform size and shape. A few were swollen and deformed, a description often seen in other haemogregarines and described as a process of degeneration (Ball *et al.*, 1967).

Due to the high prevalence of *Hepatozoon* infections in *P. gunnii* in areas commonly frequented by other animal species, it was likely that this parasite was not restricted to *P. gunnii*. No reports of *Hepatozoon* spp., from any Tasmanian marsupial had been described from Tasmania and the extended investigation by Lê, (1996) showed that in Tasmania, *Hepatozoon* spp., gametocytes are found in the Southern Brown Bandicoot (*Isoodon obesulus*) and the Swamp Rat (*Rattus lutreolus*). Host specificity is generally considered to be directly related to the validity of species

assignment for this genus (Ball *et al.*, 1967). At intermediate host level, host specificity may be diverse, as various protozoa belonging to the genus *Hepatozoon* have been found to parasitise more than one vertebrate host (Ball *et al.*, 1967; Clark *et al.*, 1973). Lê, (1996) did report that the appearance of the *Hepatozoon* gametocytes in each of these host species was markedly different, in particular the staining characteristics and dimensions. Intracellular forms were noted exclusively in the leukocytes of the Swamp Rat, as opposed to infection of erythrocytes in *P. gunnii* and *I. obesulus*.

A difference in the dimensions of gametocytes from the animals observed by Lê, (1996) was evident from the mean values obtained. This supports the hypothesis that the *Hepatozoon* parasite in *P. gunnii* may be a new species. Further comparison of this species with *Hepatozoon peramelis* from the Northern Brown Bandicoot of Queensland, revealed that the dimensions of gametocytes in *P. gunnii* were consistently lower (Lê, 1996).

In view of the data presented and work originally described by Welsh and Dalyell (1909) it seems logical to provisionally identify the parasite as a new *Hepatozoon* species. The problem in differentiating *Hepatozoon* species from those within the genus *Haemogregarina*, based solely on gametocyte morphology and morphometry of gametocytes in the blood has resulted in much taxonomic confusion (Desser, 1990). There have been suggestions by Desser (1993) that erythrocytic merogony may be a useful criterion for differentiating *Hepatozoon* species from *Haemogregarina* species (cited in Davies, 1995b). Levine (1982), attempted to bring some order to the Family Haemogregarinidae, introducing new names to replace homonyms or previously unnamed species. Unfortunately, the life cycles and vectors of the majority of species are unknown and assignment to any of these genera is often tentative as is the current case.

The sporogonic stages in the definitive (invertebrate) host of other species, specifically the size of the oocysts and the presence or absence of the sporocysts, is the primary criterion for differentiating the two genera (Desser, 1990). Haemogregarines have a heteroxenous life cycle that alternates between an intermediate vertebrate host and a definitive invertebrate vector. Although it is believed that arthropod ectoparasites act as vectors of the genus *Hepatozoon*, examinations of ixodid ticks, mites, and fleas from infected tropical bandicoots in Queensland and northern NSW have failed to reveal life-cycle stages (Mackerras, 1959a). Furthermore, the fact remains that less is known of the life-cycles and modes of transmission of *Hepatozoon* species in temperate vertebrates than of related species of blood parasites in tropical and subtropical countries (Krampitz, 1981). Ticks and fleas were collected from *P. gunnii* in the wild, but dissection and histological sections failed to reveal any evidence of life-cycle stages.

Lê, (1996) reported the *Hepatozoon* parasitaemia found in the Tasmanian *P. gunnii* existed at low levels usually lower than that found in *I. obesulus*. The highest parasitaemia observed in *P. gunnii* were 2 parasites per 1000 erythrocytes, therefore approximately half of the animals examined carried a low parasitaemia.

The detection of trypanosomes in *P. gunnii* in this study and that extended by Lê to include *I. obesulus*, was the first record for such parasites in marsupials from Tasmania. The original description of *Trypanosoma thylacis* was reported by Mackerras (1959a) from *Isoodon obesulus* captured in Queensland. Results by Lê, (1996) revealed a significant difference in size of trypanosomes detected in the two different bandicoots. This indicates the possibility of two separate and distinct species. The only other record of a trypanosome from native mammals in Tasmania is that of *Trypanosoma binneyi* (Shaw) described from the platypus (*Ornithorhynchus anatinus*) (Mackerras, 1959a).

Haematological results of *P. gunnii* infected with *Hepatozoon* sp., revealed no common trend in values that greatly deviated from calculated "normal" ranges. There appeared to be no significant differences between male or female *P. gunnii* infected with this parasite. Red blood cell counts for all infected animals showed no irregularities suggesting no red cell destruction was occurring, a phenomenon often seen in intra-erythrocytic protozoal infections and other conditions. Alternatively, the result is due to the low parasitaemia levels detected in most animals. Leishman's and Leishman's / Wright's were commonly used to stain slides and both highlighted the morphological features of the parasite well. Acridine Orange O stain did not provide great detail but placed much less strain on the observer than the Romanowsky stains for screening purposes. The attempt of isolating and maintaining *Hepatozoon* in culture proved successful. This method appears to be a good alternative to concentrating gametocytes for TEM processing and perhaps for future investigations involving molecular studies.

Generally, haematological values obtained were within the normal parameters but high white cell counts were often detected in individual animals with no clinical reason. A concurrent infection could have been a contributing factor for the high leukocytosis in these animal. Reports of dogs infected with *Hepatozoon canis* show a large increase in leukocytes with a predominance of neutrophils (Craig *et al.*, 1978), but the merozoites of this species invade leukocytes whereas the species detected in *P. gunnii* infects red cells. Obviously a comparison is difficult.

Many of the ultrastructural features identified in this study and by Lê, (1996) were consistent with those observed in other apicomplexan parasites (Desser and Weller, 1973; Scholtyseck, 1973; Bashtar *et al.* 1984), including the pellicle, conoid, the polar ring, possible rhoptries, micronemes and electron-dense bodies. Mitochondria were seen whereas micropores were not observed in any of the sections made. On the basis of electron microscopic observations it does appear that the species of

Hepatozoon shares many similarities to other *Hepatozoon* species. Many of the structures identified were found to be consistent with those observed in other Apicomplexa.

SEM was used to visualise any structural deformation in infected erythrocytes or changes to its membrane. Good cell membrane integrity and structure was observed in samples following the technique described by Lê, (1996), of processing by critical point and air drying for both sonicated and non-sonicated forms. Distortion of erthrocytes was seen in heavily parasitized animal samples, and Lê, (1996) confirmed these findings by describing cells with projections, suggesting it was highly probable that an intracellular parasite was present.

The spleen, as a secondary lymphoid organ, is an important contributor to the protective immune responses to blood-borne protozoa. This includes infections with tick-borne babesias, trypanosomes, malaria parasites and other protozoan parasites of erythrocytes (Phillips, 1996). Understanding the role of the spleen in these instances is largely based on experimental model systems, particularly in human infections. In many circumstances the degree to which extrasplenic sites can take over responsibility for effective immunity if the spleen is removed or absent, depends on the parasite, the immune status of the host (Phillips, 1996) and the animal species itself.

Observations post-splenectomy of *P. gunnii* revealed no rise in parasitaemia and no adverse affect on the animal's general health in captivity. No clinical signs or symptoms, loss in body weight, changes in body temperature or behaviour were reported. No rise in parasitaemia was observed after weekly cortisone injections. This is in contrast to observations of splenectomised raccoons infected with *Hepatozoon procyonis* by Clark *et al.*, (1973). The animals in the latter study displayed a threefold increase in circulating gametocytes 10 days post surgery. The

further increase following corticosteroid administration suggested that the removal of the spleen and corticosteroid cause immunosuppression allowing an increase in schizogony. The mechanism by which splenectomy and corticosteroid administration temporarily increased the numbers of circulating gametocytes has not been determined.

With regard to *P. gunnii*, the spleen does not appear to play a significant role during the course of *Hepatozoon* infection or at least in well established infections. The role of gametocyte removal may be taken up by macrophages from the liver. It appears that the immune status of the host is therefore reasonably complete or the spleen provides a major reservoir for developing schizonts. An histological examination of the spleen failed to detect the presence of these forms suggesting that the latter view may not be true. In fact, no life-stages of *Hepatozoon* were seen from the histology of any of the organs in *P. gunnii*, as opposed to the study by Clark *et al.*, (1973) who reported typical lesions in the myocardium of the raccoon infected with *H. procyonis*. These lesions in the raccoon were somewhat similar to those seen in animals suffering from American trypanosomiasis (*Trypanosoma cruzi*) and toxoplasmosis. Clark *et al.*, (1973) reported no evidence of inflammation or cellular damage associated with immature schizonts but the intense inflammatory response was associated with the release of merozoites with marked lesions characterised by accumulations of macrophages containing phagocytized organisms.

An initial rise in the concentration of platelets and leukocytes was found in post-splenectomy smears of *P. gunnii.* This feature seems to be a common presentation of post-splenectomy in humans (Beck, 1992). By day 25, a decrease in mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) values, and (PLT) platelet counts had decreased which often reflect post surgery bleeding. In humans a decreased MCHC in combination with a low mean

corpuscular volume (MCV) indicates a microcytic-hypochromic anaemia (Beck, 1992).

Observations of previously captured *P. gunnii* infected with *Hepatozoon* were monitored over a 10 month period. After 3 months gametocytes were no longer found in blood smears and remained negative until the end of the project. Clark *et al*, (1973) described periodical appearances of gametocytes in raccoons. Similarly seasonal periodical appearances of *Hepatozoon canis* gametocytes in the peripheral blood of dogs were reported by Murata *et al.*, (1993). It was proposed by Murata *et al.*, (1993) that the appearance of the gametocytes in the blood synchronized with the seasonal activity of the tick vector. In *P. gunnii* it is likely that the appearance of gametocytes belonging to the genus *Hepatozoon* is subject to some form of periodicity in the wild due to the activity of the arthropod host. Another possibility is that infection occurs, culminating with the production of long-lived gametocytes and disappearance of all other stages. This would explain all the findings.

The true significance of hepatozoonosis on the wild *P. gunnii* population remains to be determined, due to the fact the animals observed for this investigation were held in captivity on a regular diet without constant reinfection by the vector host and in the absence of other diseases. The recognition of this disease remains significant, however, as it may be an important predisposing factor rendering *P. gunnii* more susceptible to other diseases such as toxoplasmosis. Concurrent *Hepatozoon* diseases and *T. gondii* have been reported in dogs (Harmelin *et al.*, 1992), while the over-representation of cats with a retroviral disease among cats with hepatozoonosis, indicates an association between immunosuppression and the development of *Hepatozoon* infection (Baneth *et al.*, 1998). The same may be true for trypanosomes, where again infected animals did not appear to be clinically affected by the infection in the field or laboratory (Lê, 1996).

Knowledge of these aspects of haemogregarine biology is essential for the determination of the taxonomic status of the protozoa described in *P. gunnii.* Unfortunately due to the limitations of working with a "vulnerable" species the results of splenectomy are based on the observations of only one animal. The clinical significance of both the *Hepatozoon* sp., and the trypanosome thus remains unknown, as does their potential role in contributing to the marsupial's susceptibility to other infections. The large number of Tasmanian *P. gunnii* found infected by *Hepatozoon* sp., and the presence of trypanosomes in the bandicoots, warrants an attempt to identify the mode of transmission, life cycle and provide taxonomic classification of these organisms.

Chapter 5. *Giardia* in *P. gunnii* and Tasmanian Waters

5.1 INTRODUCTION

Goldsmid and Wellock, (1984) first demonstrated that *G. duodenalis* was an important intestinal pathogen of humans in Tasmanian communities, with strong suggestions of familial transmission. In preliminary investigations, *G. duodenalis* was detected from 2-3% of patients suffering from diarrhoea statewide (Goldsmid, 1980, Goldsmid, 1981, Hawkesford and Goldsmid 1995). Epidemic outbreaks of the infection are thus not surprisingly recorded from time to time in daycare centres and similar environments that are conducive to facilitating person-to-person transmission (Goldsmid and Wellock, 1984). The prevalence of *G. duodenalis* infection is even higher in refugees, immigrants, and returned travellers from developing countries to Tasmania, reported levels being as high as 11% (Goldsmid, 1979, Goldsmid, 1988, Paget *et al.*, 1995).

The first case involving connected giardiasis of an animal and its owner in Tasmania, was described by Davies *et al.*, (1993). This was followed by a general survey of *Giardia* infection in dogs and cats in Tasmania, with prevalences reported to be as high as 19% and 20% respectively (Milstein and Goldsmid 1995; Kettelwell *et al.*, 1998). A survey by Davies, (1995a) of the zoonotic potential of native animals in Tasmania found 5.5% of Tasmanian native animals were infected with *Giardia* spp. A comprehensive survey by Kettlewell *et al.*, (1998) found 21% of native animals were infected with *Giardia* which raised the questions, what is the prevalence *Giardia* in *Perameles gunnii*? What is the source of their infection? What, if any, clinical effects result from this infection? And are bandicoots involved as a reservoir of infection for humans?

Animals have been implicated as an important zoonotic source of human giardiasis (Wallis *et al.*, 1984; Sautter and Knights, 1983), though humans may also

contribute to the source of cysts in the environment. The interaction of humans and the wilderness in Australia has since European settlement, resulted in numerous repercussions on our fauna and flora. This raises the question of whether humans could serve as a source for transmission of *Giardia* to wildlife of Tasmania?

Tasmania is a temperate island state with mountains, lakes and forests, with almost 30% of its landmass designated as World Heritage Area, national parks and reserves. It remains economically reliant on its primary produce, but for several years now its tourist industry has flourished, with the island becoming one of Australia's most diverse holiday destinations. As its most attractive feature, Tasmania offers a wide variety of wilderness activities used by locals and overseas visitors, the most popular of which is bushwalking through wilderness areas. As a holiday destination Tasmania appears to offer a safe environment due to its pristine image, but its reputation for keen bushwalkers is often marred by "Bushwalker's Diarrhoea" for which *G. duodenalis* is often blamed, although there is little laboratory data to justify this claim. At one stage, Tasmania was classified as an area of moderate risk or unknown status for the acquisition of Travellers' Diarrhoea (Dupont and Pickering, 1980). This claim was refuted by Goldsmid, (1991) but despite this, *Giardia* infection is a problem within the State. Tasmania's Parks and Wildlife Service has fervently implemented and supported the Threatened Species Strategy and Natural Heritage Trust, to maintain vigilance on the environment and its species while encouraging the use by visitors. Unfortunately, human behaviour in the wilderness is questionable, as implementation of hygiene guidelines set out before entering a Heritage Area or reserve are not always strictly adhered to (Driessen, personal communication).

The epidemiology of human giardiasis is often the subject of debate, focussing on the potential role of wild mammals as a source of infection. There is no doubt that small mammals can be infected with human *Giardia.* Examples include Sehgal *et*

al., (1976), and Craft, (1982) who succeeded in infecting adult and weanling rats with cysts of human origin and Hewlett *et al.*, (1982) who experimentally infected mongrel dogs with cysts and cultured trophozoites. The dogs were not clinically ill but the result supported the concept of interspecies transmission of *Giardia* and the study demonstrated that infection can be transmitted by the trophozoite form as well as by the cyst form. Belosevic *et al.*, (1983) and Rendtorff, (1954) clearly demonstrated that *G. duodenalis* was a true infection of animals with clinical symptoms and pathology similar to the human disease. A human volunteer and Mongolian gerbils were shown to be susceptible to *Giardia* isolated from a rat (Majewska, 1994). Animals can therefore become reservoirs, amplifying further transmission to humans and to other animals.

What is the risk of *Giardia* infection in native animals? Feral cats for instance have received great attention in the past for being predators, but are possible disease reservoirs for wildlife such as *P. gunnii* (Obendorf and Munday, 1990; Driessen and Hocking, 1991). The incidence of *Giardia* in populations of feral cats is reportedly as high as 21% and for other protozoa such as *Toxoplasma gondii* the incidence may be as high as 50% (Milstein and Goldsmid, 1995). What implications can *Giardia* have on marsupials such as *P. gunnii*? An experimental investigation was performed to detect the effects, if any, on *P. gunnii* inoculated with *G. duodenalis* cysts isolated from a human patient.

For omnivorous and insectivorous marsupial species such as bandicoots, a possible source of *Giardia* infection may be ingestion of soil-associated arthropods, insects or annelids such as earthworms that have come in contact with cysts in faeces of infected animals. After exposure to contaminated soil, plant material or faeces, such invertebrates may act as mechanical hosts by harbouring viable cysts within their gastrointestinal tract as has been shown for *T. gondii* (Frenkel *et al.*, 1975; Ruiz and Frenkel, 1980; Bettiol and Obendorf, 1997). Based on dietary evidence

available it was speculated that *P. gunnii* might acquire infection through ingestion of soil-associated oligocheates (earthworms) and arthropods containing infective stages of *Giardia*.

Are Tasmania's waterways a potential risk to humans and animals? What then is the risk of *Giardia* infection for visitors to Tasmania, and what external factors are involved? As part of an ongoing investigation of the epidemiology of giardiasis in Tasmania, native animals including *P. gunnii* and water samples were investigated for *Giardia* using a range of parasitological detection techniques. Water samples were also screened for *Cryptosporidium* which is recognised as an important enteric protozoan pathogen world-wide (Faulkner *et al.*, 1995). *Cryptosporidium parvum* is a common cause of diarrhoea in the human population of Tasmania. Hawkesford, (1986) recorded a prevalence of 2% in patients with diarrhoea. But the parasite has been reported to cross host barriers, with domestic animals and pets acting as reservoirs (O'Donoghue, 1984; Milstein, 1993). Similarly, Davies, (1995a) reported 15% of Tasmanian native animals surveyed were infected with *Cryptosporidium* suggesting a potential role as reservoirs of this parasite. Though no *P. gunnii* have been reported with *Cryptospordium* to date, the investigation of both *Giardia* sp. and *Cryptosporidium* from Tasmanian waterways was necessary.

5.2 MATERIALS AND METHODS

5.2.1 General Methods

Parasitological examination of stools involved general methods as described in Chapter 2; 2.7. Faecal specimens were examined for the presence of *G. duodenalis* using direct wet mount and a combination of Wheatley Trichrome stain, formalin-ethylacetate sedimentation concentration (Garcia and Bruckner, 1993), using Johns Parafilter System (Biolab Scientific Pty. Ltd), and the Zinc Sulphate Flotation Method (Smith and Bartlett., 1991). Considering the success rate for microscopic detection (gold standard) of cysts from a single faecal specimen is only 50-70% all samples were further tested for specific *Giardia* antigen using the commercial antigen detection kit *Giardia* CELISA enzyme linked immunosorbent assay (ELISA), (Cellabs Diagnositics Pty. Ltd. Brookvale, Australia).

5.2.2 *Giardia* CELISA Kit.

All specimens were processed in accordance with the manufacturer's instruction (Appendix 11.5a). The method detects *Giardia* antigens in a capture enzyme immunoassay. Antigens from faecal specimens are bound to microplates which have been coated with purified mouse monoclonal antibodies to *Giardia*. Enzyme activity is directly proportional to the concentration of *Giardia* antigen present in the sample and control. Once the reaction is completed the plates are read using a microplate reader with a 450 nm wavelength filter. The cut-off is calculated in accordance with the test instructions, by adding 100% to the mean of the absorbance of the negative controls. Any specimens with absorbance values greater than the cut-off value are considered positive. Specimens with absorbance values less than, or equal to the cut-off value are considered negative.

5.2.3 Inoculation of *P. gunnii* with *Giardia* cysts: Experiment 5.1

In 1995, four Eastern Barred Bandicoots (*Perameles gunnii*) captured at Huon valley in southern Tasmania were used in this study. Animals were caught in wire

cage traps with a permit to take protected wildlife (permit numbers 95249 and 95256). The marsupials were housed in the Central Animal House at the University of Tasmania (University Ethics Committee, Animal Experimentation; Investigation number 95082).

All animals were initially placed in single-animal holding cages, each with a nest box. All animals were acclimatised to their environment and food source for over a month prior to the experiment. After this period all animals were transferred from the original holding pens to sterilized enclosures. Care was taken at all stages of food handling and preparation to avoid contamination from any infectious source and sterile water was provided *ad libitum*.

Examination of daily stools over ten days confirmed that the animals were healthy and *G. duodenalis* free. All animals were screened for the presence of *G. duodenalis* using a combination of techniques, including a direct wet mount and combination of Wheatley Trichrome stain, formalin-ethylacetate sedimentation concentration, Zinc Sulphate Flotation Method, including antigen detection using *Giardia* CELISA. Care was taken to ensure that no antibiotics were administered prior to the experiment, as a precaution to unforeseen complications. All animals were weighed before and after the experiment. Two animals were inoculated orally with cysts from a human case of *G. duodenalis* and two animal controls were maintained uninfected.

The *G. duodenalis* cysts were isolated from a human patient with chronic giardiasis. The faecal material was mixed with a small volume of Phosphate Buffered Saline and Triton X-100 (Mallinckrodt, Kentucky, USA) and filtered using parafilters and rendered free of faecal matter. It was then centrifuged at 500 x *g* with benchtop centrifuge (Sorvall RT, 6000 D, DuPont Ltd, Delaware, USA) for 5 minutes, three times. Each time the supernatant was discarded. At the end of a fourth spin the pellet was resuspended in 5 mL of normal saline and 0.5 mL Mefoxin (100 mg,

equivalent to cefoxitin 2 g). The solution was left standing for 5 minutes and then centrifuged at 500 x *g*. This solution was again resuspended in 10 mL of normal saline.

One mL of the *G. duodenalis* cyst suspension (150 cysts / mL) collected from the human case of giardiasis was diluted with 2 mL of sterile distilled water. Three mLs of inoculum was administered *per os* to each animal. The suspension was carefully deposited directly in the oesophagus of a restrained animal by use of a canula. The inoculated marsupials and control animals were housed in sterilized wire cages. Stool specimens were processed and examined for the *G. duodenalis*. Bedding material was removed each day and incinerated. The animals were weighed, observed and their body temperature taken by digital thermometer inserted into the rectum daily.

Specimens were examined for the presence of *G. duodenalis* using the concentration techniques and permanent staining techniques. Concentrated specimens were examined microscopically with a drop of iodine. When using the *Giardia* CELISA commercial kit, all specimens were prepared and tested according to the manufacturers guidelines.

5.2.4 Earthworms and soil conditions: Experiment 2.2

Earthworms used were commercially reared Red earthworm *Lumbricus rubellus* and the Blue earthworm *Perionyn excavatus* (All State Worm, P/L, Adelaide, South Australia). Earthworms were purged over a 3 day period prior to experimental work. Soil treatment and earthworm maintenance is described in Chapter 2; 2.6. Following these procedures and when purged, approximately 100 worms, were divided equally between two plastic boxes containing fresh soil. The soil was enriched with a protein mix of eggs and sterile milk powder once every 2 weeks to provide nutrient for the worms. One group was maintained in uninfected soil as the

control group, the other group was placed in soil infected with *Giardia* cysts of human origin.

A suspension of viable *Giardia* cysts was mixed in 25 mL of distilled water and dispersed onto the soil surface. The sample was delivered with a hand held spray. The surface was gently mixed and left for one week to ensure that the worms were fully exposed.

5.2.5 Collection and Preparation of Water Samples for the detection of *Giardia* cysts and *Cryptosporidium parvum* oocysts.

Due to commercial reasons and agreement with Macquarie University and the Sydney Water Board, the water concentration techniques used for *Giardia* detection in this thesis cannot be described in full.

Water samples from rivers across Tasmania were collected in specially prepared large mouth polypropylene sample bottles in 20 to 40 L lots. Samples collected were from the following rivers and regions; Arve River (Picton), Esperance River (Waterloo), Junee River (Maydena), Little Denison River (Lonnavale), Lawrence River (Gordonvale) and four river samples from Pelion region. All the rivers were situated in the south or south-western regions of Tasmania. Most were in remote, non-camping areas where there is little if any human contact or farming. The Pelion region, even though situated in a Heritage Area in Tasmania's far South-West, has however become a major thoroughfare for bushwalkers and campers. Four samples from this region were airlifted out at the end of a summer season.

Samples were concentrated using a Calcium Carbonate flocculation technique developed by Vesey *et al.*, 1993 (adapted by Dr R Shanker, Macquarie University). This procedure is used to concentrate highly turbid (>10 NTU) samples of less than 40 L. The addition of sodium bicarbonate and calcium chloride combines to form calcium carbonate floc within the sample, concentrating cysts into a dense residue

within 4 – 24 hours. 'The addition of Sulphamic acid (BDH, Poole, England) quickly and effectively dissolves the floc leaving a clear suspension of cysts and debris. Further concentration is performed using Tween-20 with formalin and combined washing steps. The sample is then Pre-filtered using a range of wire mesh sieves ranging from 38 µM to 100 µM (Endecotts Ltd, London, UK) to remove any large pieces of sediment or fibrous material which may interfere with later flow cytometric analysis. The sample is then prepared for flow cytometry and immunofluorescent staining

2.10.1 Preparation of Water Samples for Flow Cytometry

Centrifuge sample in the 15 mL centrifuge tube at 800 x *g* for 10 minutes at 4°C ± 2°C. Remove most of the supernatant, leaving enough to resuspend pellet. Vortex the sample and remove an aliquot no larger that 500 µL, expressing this as a percentage of the total sample volume (25%, 50%, 100%). Place aliquot in a 12 x 75 mm sample tube and weigh. Add 10% (w/v) sample buffer to the sample. Cover the tube (Falcon 12 x 75 mm, Becton Dickinson and Company, New Jersey, USA) with parafilm and vortex for 5 to 10 seconds. Label. At this stage the sample may be stored at 4°C ± 2°C for several weeks, prior to immunofluorescent staining.

5.2.5.1 Immunofluorescent Staining (Chapter 2; 2.10)

Direct Fluorescent Antibody Procedure for detection of *Giardia* cysts and *Cryptosporidium* oocysts was performed using commercial antibody. Methods were performed following manufacturers instructions. The antibodies used to label samples in this study were supplied in a kit form and included A100 AquaGlo G/C Direct NSW, (Waterborne, New Orleans, USA) and A300Fl. Giardi-a-Glo (Cellabs, NSW, Australia). The first kit is a mixture of FITC-labelled monoclonal antibodies made against cysts of *Giardia lamblia* (= *G. intestinalis, =G. duodenalis*) and oocysts of *Cryptosporidium parvum* as 1 mL of a 20X-concentrated solution.

A300Fl., Giardi-a-Glo contains fluorescein-labelled IgG mouse monoclonal antibody made against cysts of *Giardia lamblia.* Antibody reagent was diluted by 20-fold with Dilution /Blocking Buffer supplied with the kit, to give the required volume of 1X working dilution. 100 µL of monoclonal antibody was added to 100 µL of concentrated water sample and incubated at 37°C for 30 minutes before analysis.

5.2.5.2 Flow Cytometry utilising Fluorescence Activated Cell Sorting (FACS)

Flow cytometric analysis was performed on a Coulter Elite ESP (Coulter Corporation, Pty Ltd, Florida, Miami, USA) by using a 488-nm line of an argon ion laser. Green fluorescence was collected by using a 550 nm dichroic and 525-nm band pass. Data was analysed using Elite Software version 4.02. Non-fluorescent debris was excluded using forward and 90 degree scatter. Samples were analysed directly after labelling with monoclonal antibody. Other samples were fixed with Permafluor Aqueous Mounting Medium Liquid (Immunotech, Cat no. 0752, Marseille, France) and scanned using fluorescent microscopy. Images were captured using image analysis.

5.3 RESULTS

5.3.1 *Giardia* in Tasmanian marsupials

Of the 31 stool samples processed, 55% (n=17) were detected with *Giardia* (Appendix 11.5). Four were positively identified using all procedures outlined that included microscopy and antigen detection (Table 5.1). Twelve were reported positive using the antigen detection kit and three samples were identified as positive with microscopy alone. The combination of techniques utilised, emphasises that sensitivity of using antigen detection with the "gold standard" parasitological techniques increases the detection of *Giardia*, especially when dealing with animal specimens.

Giardia detection in *P. gunnii*	
No. *P. gunnii* examined	31
No. positive – Microscopy only	2
No. positive – CELISA	12
No. positive – Microscopy and CELISA	3
Total No. positive	17

Table 5.1 Results of screening *P. gunnii* faecal specimens for *Giardia* using microscopy, antigen detection (CELISA) and a combination.

P. gunnii	Clinical Signs	*Giardia* spp.
Experimental animal 1	+	+
Experimental animal 2	-	-
Control 1	-	-
Control 2	-	-

Table 5.2 Summary of results following experimental inoculation of *P. gunnii* with human *Giardia* cysts.

5.3.2 Experimental *Giardia* in *P. gunnii*.

The study of experimentally infecting *P. gunnii* of human origin indicated a susceptibility to infection with *G. duodenalis* from a human source. Ingestion of cysts produced infection in one of two experimentally infected bandicoots, with a prepatent period of 9 days. Cyst excretion was continuous until 20 days post infection. Identification of cysts was performed using both parasitological and antigen detection techniques. *Giardia* cysts were permanently stained using the Wheatley Trichome stain or Gomori stain (Figure 5.1). The faeces of the infected animal were intermittently pale and loose/unformed but not throughout the course of cyst excretion. Diarrhoea was not detected in the second animal used in this study. No weight loss was detected in either animal and no body temperature changes were detected during the course of the infection. No haematological changes were evident during the experiment. Based on both parasitological and antigen detection techniques, the two control animals remained free of *G. duodenalis* and other intestinal parasites during this period. Results summarised in Table 5.2. When the experimentally infected animals were considered to be *Giardia*-free, they were released back to their native habitat.

5.3.3 Earthworm a source of *Giardia* for *P. gunnii*

Giardia cysts were detected in the soil samples using microscopy and zinc sulphate method. Earthworms exposed to the soil were macerated and scanned for *Giardia* cysts using the range parasitological microscopic techniques described Chapter 2 but no *Giardia* cysts were detected. These samples were also tested using the *Giardia* CELISA kit but due to cross-reactivity and results in control animals indicated were reported as false-positives. Earthworms were preserved in formal saline and sectioned for histology and stained using H & E. No cysts could be detected in the earthworm sections.

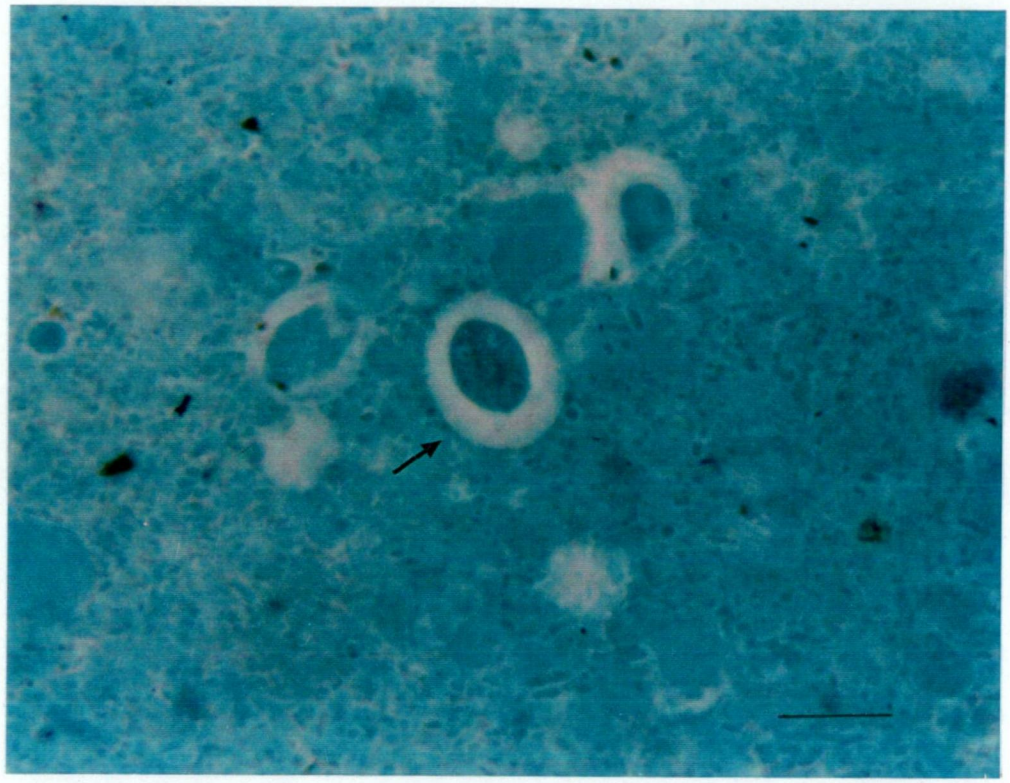


Figure 5.1. Cyst of *Giardia* isolated from faecal concentrate with visible halo (arrow) resulting form cyst shrinkage during permanent staining.

Permanent Stain: Wheatley Trichome

Bar = 10 μM

5.3.4 Water as a route of *Giardia* Infection in Tasmania

Ten water samples were processed for detection of *Giardia* cyst and *Cryptosporidium* oocysts. Final processing involved immunofluorescent labelling with commercial monoclonal antibodies and examination by flow cytometry. Samples were electronically gated and analysed for fluorescence intensity. Analysis of acquired data was presented as Fluorescence (FITC) versus Forward Scatter (FS) diagrams.

Analysis of scatter diagrams of all waters screened revealed only samples from the Arve River contained both *Giardia* cysts and *Cryptosporidium* oocysts (Table 5.3). The Arve River samples were electronically gated with Region 1 (R1) representing the profile of *Cryptosporidium* oocysts and Region 2 (R2) representing the gated region for *Giardia* cysts (Figure 5.2 (a)). All four samples from the Pelion region contained *Giardia* cysts. Pelion water samples were gated with Figure 5.2 (b) representing regions (R2) detected, suggesting the presence of *Giardia* cysts. All other water samples processed and analysed using flow cytometry did not display detectable regions to suggest the presence of *Giardia* cysts or *Cryptosporidium* oocysts and were considered negative. Figure 5.2 (c) is a representative scatter diagram of these samples.

Sorting of particles by flow cytometry was rapid, but as most samples contained particles that could mimic oocysts and cysts in size it was essential that fluorescent microscopy be performed for confirmation and to exclude any false positive results. Detection of cysts by microscopy was confirmed by size, shape and internal morphological characteristics and captured using image analysis. Figure 5.3 demonstrates *Giardia* cyst and *Cryptosporidium* oocyst isolated from the Arve River and labelled using fluorescein-labelled dual monoclonal antibody cocktail. Image was captured using image analysis.

Water Source	*Giardia* spp.	*Cryptosporidium parvum*
Arve River (Picton)	+	+
Esperance River (Waterloo)	-	-
Junee River (Maydena)	-	-
Little Denison River (Lonnavale)	-	-
Russel River (Lonnavale)	-	-
Lawrence River (Gordonvale)	-	-
Pelion	+	-
Pelion	+	-
Pelion	+	-
Pelion	+	-

Table 5.3 A survey of Tasmanian waterways for the presence of *Giardia* cysts and *Cryptosporidium parvum* oocysts using FACS analysis.

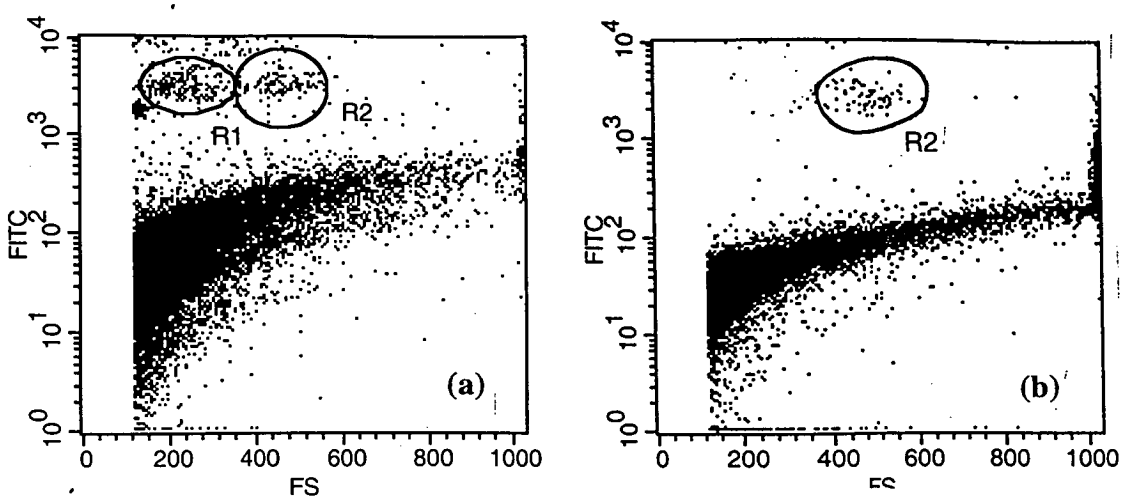


Figure 5.2 (a) and (b). Tasmanian water samples were processed and labelled with immunofluorescent monoclonal antibodies and analysed for fluorescence intensity using flow cytometry. Acquired data are presented as Fluorescence (FITC) versus Forward Scatter (FS) diagrams. Regions R1 and R2 represent the detection of *Cryptosporidium* (R1) and *Giardia* (R2). Figure 5.2 (a) shows the presence of *Cryptosporidium* (R1) and *Giardia* (R2) in the water sample from Avre River. Figure 5.2 (b) represents the detection of *Giardia* (R1) in water samples from the Pelion.

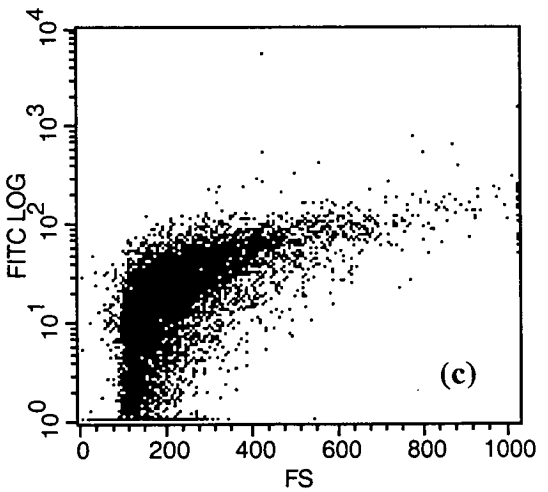


Figure 5.2 (c). Represents water samples with no detectable regions to suggest the presence of *Giardia* cysts or *Cryptosporidium* oocysts and were considered negative

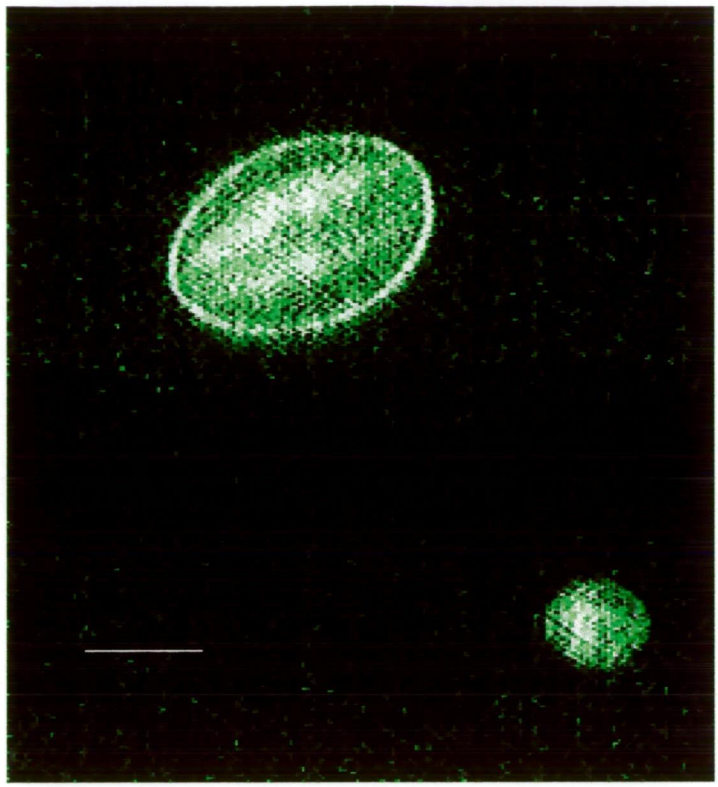


Figure 5.3 The ovoid *Giardia* cyst and the smaller round *Cryptosporidium* oocysts isolated from the Arve River and stained with fluorescein-labelled monoclonal antibodies and detected using image analysis.

Bar = 5 μM

5.4 DISCUSSION

The diagnosis of giardiasis is usually made by the gold-standard method of microscopical examination of the faeces. Although the specificity of the method is 100%, cysts are excreted intermittently or may be present in numbers too low to be detected in infected individuals. The sensitivity of this method is thus rather poor. Diagnosis of giardiasis using microscopic methods can sometimes be difficult due to the morphology of the organisms, and this was true for cysts isolated from *P. gunnii*. The cysts often appeared dehydrated and slightly misshapen with faint internal structures. The possible reasons for this are the difficulties in collecting fresh specimens in the wild and by the time the collected faecal specimens reach the laboratory the specimens can be 1 to 2 days old. The accuracy of stool microscopy is often dependent on the examination of repeat specimens, a situation that is difficult to achieve when dealing with animals in a capture and release program. For these reasons, the microscopic parasitological techniques in this investigation were supplemented with the *Giardia* CELISA kit for detecting *G. duodenalis* antigen in faecal samples. In humans the ELISA test for *Giardia* has been shown to have a sensitivity of 97% with a specificity of 96% (Rosenblatt *et al*, 1993). Hopkins *et al.*, (1993) demonstrated the kit had a specificity between 91 and 95%, with a sensitivity of 64% when used to detect antigens of other animals species. This being the case, it seemed that a screen of native animals using the *Giardia* CELISA would more likely underestimate the rate of infection in these animals than give an exaggerated picture.

The recorded prevalence rate of 55% in bandicoots infected with *Giardia* was high for a native animal population and compared to 6% within wild populations of Tasmanian Devil and other Dasyuridae, using the same detection techniques (Kettlewell *et al.*, 1998). The prevalence of *Giardia* in companion animals in Tasmania such as dogs and cats, is reported to be 19% and 20% (Kettlewell *et al.*, 1998).

The experimental infection of captive *P. gunnii* with *Giardia* cysts isolated from human faeces highlights the possibility of free-ranging native animals becoming infected with *Giardia* of human origin. There would probably be few *Giardia* investigators today who would refuse to accept that human *Giardia* infections can be successfully established in other selected animal species. In this study, only 150 cysts isolated from a human source were required to establish an infection in *P. gunnii*, with symptoms of diarrhoea. Work by Belosevic *et al.*, (1983) demonstrated that the Mongolian gerbil could readily support the establishment of *Giardia* infections from cysts derived from several host species. Erlandsen *et al.*, (1988) provided evidence of infections in beavers and muskrats by using variable doses of viable cysts.

There are reports of spontaneous resolution of *Giardia* infection in both experimental animals and humans, as was noted in *P. gunnii* in this investigation. Faubert, (1996) considered prior exposure confers some resistance to reinfection. The existence of a protective immune response to *Giardia* has been known for some time (Faubert, 1996) but this may be countered by antigenic variation by the parasite as discussed by Mills and Goldsmid, (1995). Questions that still remain unanswered refer to the strain of *G. duodenalis* infecting bandicoots. It is not known at this point if the naturally occurring strains of *Giardia* in the bandicoots are identical to those infecting humans, or how the *P. gunnii* initially became infected. There is the possibility that human intrusion and defaecation in wilderness areas are a source of infection to native animals. However, it is possible native animals were infected prior to human encroachment into these areas and an indigenous strain/species exists. This suggests that humans can be infected by *Giardia* from native animals, including *P. gunnii.* In particular, it is necessary to culture and identify the strains of *G. duodenalis,* and further genetic studies may resolve much of the confusion. Recently, Hopkins *et al.*, (1997) found differences between the genotypes of *Giardia* isolates recovered from humans and dogs living

in the same locality in Western Australia, suggesting a new *G. duodenalis* subgroup while a novel genotype of *Giardia* in a Southern Brown Bandicoot is likely (Adams, personal communication). Further work on native animals in this area is essential to resolve this intriguing situation.

Transmission of giardiasis occurs predominantly via the faecal-oral route, when cysts are ingested from food or water contaminated by infected human or animal faeces (Thompson *et al.*, 1993). During the course of infection, people and animals infected with *Giardia* excrete enormous numbers of cysts and it is similarly possible that *P. gunnii* obtain their infections from human cysts in the water or other environmental sources. Bandicoots are not normally seen drinking water in the wild but their digging behaviour and diet preference for coprophagic invertebrates such as earthworms usually provide their source of water (Heinsohn, 1966). It has been noted however, that these animals do drink water in captivity and thus, they may in times of nutritional deprivation or scarcity of invertebrates, be forced to seek water in the wild, and which may contain *Giardia* cysts. In times of stress, bandicoots may well experience higher infection rates with *Giardia* than normal, particularly when food is scarce. Leitch *et al.*, (1993) describes a low dietary protein intake increases the severity and duration of infection with *G. duodenalis* in Mongolian gerbils. It was noted a reduced number of enterocytes were produced within these animals and fewer cells migrated towards the damaged villi. These changes were noted to coincide with an increase in the severity of the infection.

With the high prevalence of *Giardia* in *P. gunnii*, and reports of *Giardia* in other native animals (Davies, 1995a), it was assumed that these animals would acquire *Giardia* either from browsing on contaminated pasture or from drinking contaminated water. It was proposed that *P. gunnii* food sources, such as earthworms, were possibly acting as a paratenic host, ingesting cysts with the soil

and passing them to the bandicoots. Understanding *P. gunnii* dietary requirements and digging behaviour strongly suggests earthworms, especially, to be a potential source of *Giardia* exposure. Earthworms (Annelida) and beetles (Coleoptera) make up a significant proportion of the diet of *P. gunnii* (Heinsohn, 1966, Quin, 1985; Brown, 1989, Dufty, 1994, Mallick *et al*, 1997c). Most are soil-associated invertebrates which are coprophagic and capable of acting as mechanical transport hosts for many pathogens including *T. gondii* oocysts, through direct ingestion of faeces or soil contaminated with faeces (Markus, 1974; Frenkel *et al.*, 1975; Ruiz and Frenkel, 1980). Many animals bury their faeces in surface layers of soft soil, allowing a range of invertebrates to ingest infective *Giardia* cysts by contact directly with faeces. Ingested cysts may be carried in their gut and dispersed through their discarded alimentary casts.

Testing for *Giardia* cysts in earthworms proved difficult in our experimental model. Using the *Giardia* CELISA test, there was extensive cross-reactivity occurring with the gut content of the earthworms, even with the negative controls. Despite this, however, the effect of ingestion on the cyst is not clear. No evidence of cysts in the gut cavity could be seen on histological sections. This indicates that cysts were either destroyed in processing or not detected. The experimental design did not clearly establish the role of the earthworm but introduced the concept of soil-associated invertebrates acting as mechanical hosts in transporting *Giardia* cysts.

Davies, (1995a) and Kettlewell, (1995) reported *Giardia* in the faeces of Tasmanian bandicoots from a variety of geographical areas including, national parks, rural and urban areas. Transmission from water sources contaminated by domestic animals and farming stock is possible (Davies, 1995a), but it was unknown if water sources in remote areas of Tasmania would contain *Giardia* spp. The water samples in this survey, including the Arve River, were selected for their isolation with little or no human contact and no farm or domestic stock nearby. The Pelion region was

particularly isolated but frequented by bushwalkers on the route of the popular Overland Track walk.

The present results support the investigations of water samples by Faulkner *et al.*, (1995) who detected *Giardia* in "more Tasmanian water samples than mainland samples." The role of water in *Giardia* transmission is perhaps not surprising as the natural water in Tasmania tends to be colder than mainland states of Australia, and *Giardia* cysts are known to survive best at temperatures below 8°C (Meyer and Jarroll, 1980). Furthermore, this implies that rivers, dams, lakes and other natural water bodies in Tasmania could become ideal reservoirs of *Giardia* as could urban reservoirs even when the water is chlorinated as *Giardia* cysts are known to be resistant to standard levels of chlorination (Craun, 1990).

Viable *Giardia* cysts are infective and extremely robust, capable of surviving considerable environmental pressures. Camping areas and bushwalking tracks often provide a source of contamination to soils and waterways. Bushwalkers and campers in Tasmania are warned to boil water or use iodine tablets as the ultimate precaution before consuming water. Runoff into rivers or lakes from makeshift toilets or buried faeces following a heavy rainfall is possible, as well as animals digging close to a camp site and defaecating along river beds and waterways. It becomes a difficult task to test water supplies in wilderness areas, especially in Tasmania, when even small volumes have to be airlifted out.

Detection of *Giardia* in water samples is often difficult, as *Giardia* cysts are small enough to evade removal by filtration. Cysts are often present in low numbers, and water quality indicators such as faecal coliform counts seem to have no value in indicating the potential of *Giardia* infections from water supplies (Craun, 1990). If water bodies used for extraction of potable supplies are contaminated by faeces from infected animals, humans or sewage and wastewater discharges, there is the strong possibility of parasites entering the public water supply. This has been

reported in Australia's capital cities in recent years (Wade and Fairley, 1998) and other parts of the world (Isaac-Renton *et al.*, 1994).

Vesey *et al.*, (1993; 1994) developed a flow cytometry technique for the detection of *Giardia* and *Cryptosporidium* in environmental water that was rapid and less laborious than direct microscopical examination. The detection of *Giardia* and the occasional *Cryptosporidium* in this survey of Tasmanian waters, shows the ease in examining small water samples processed by the flocculation and concentration technique and utilising Fluorescence Activated Cell Sorting (FACS). The method does not identify the host species of origin of the protozoa, nor determine the viability status or infectivity of the detected cysts or oocysts, but the technique outlines a rapid method of detection. It does, however, allow and suggest a need for further and more thorough investigations of Tasmanian waterways in the future. The test method on a larger scale has become useful in determining the contamination of water supplies and in evaluating the effectiveness of treatment practices in a commercial setting with the possibility of quantitative values (Vesey *et al.*, 1994). There may be a need, however, to determine whether all *Giardia* are both viable and infectious to humans.

The present study has shown that native animals can become infected with human *Giardia* strains and this, together with the apparent prevalence of *Giardia* in native animal species supports the hypothesis of cross species infection. The experimental infection of *P. gunnii* with human *Giardia* cysts, elicited a transient diarrhoea in one of the animals, so the role of the marsupial as a zoonotic host of infection in nature is therefore a strong possibility. This study elicits many questions and there is enormous scope for further investigation.

Chapter 6. Other Parasitic and Microbial Diseases
A. Parasites of Free-Ranging *P. gunnii*

6.1 INTRODUCTION

Although much of the attention received by parasitic diseases in marsupials has focused on the effect these diseases may have on the regulation of native host populations, concern has been directed towards their zoonotic potential in humans and domestic stock. Amongst the marsupials most attention has fallen on macropodids such as the kangaroos and wallabies (Beveridge *et al.*, 1985), though increasing interest is being shown in small dasyurids including marsupial mice such as *Antechinus* spp., (Beveridge and Barker, 1975; Spratt, 1982; Smales, 1999). The implications of these parasites on their hosts are variable depending on their pathogenicity and circumstances. One example is *Strongyloides* spp., that may cause mild gastric ulceration in free-living kangaroos but more often cause severe disease or mortality in captive animals (Beveridge, 1988). Similarly, many macropodids harbour a diversity of *Eimeria* spp., coccidians which may cause devastating disease outbreaks when the animals are confined in captivity (Barker *et al.*, 1988a; Barker *et al.*, 1988b; Barker *et al.*, 1989). More importantly, marsupials have become hosts to the common liver fluke, *Fasciola hepatica* (Spratt and Presidente 1981; Speare *et al.*, 1989) and cysts of the hydatid tapeworm, *Echinococcus granulosus* (Coman, 1972; Speare *et al.*, 1989) reported in macropods across south-eastern Australia, except Tasmania. The health implications certainly encompass not only the well being of native fauna but the zoonotic potential to the health of humans and companion animals that may directly or indirectly, come into contact with these animals.

Several parasites and the associated pathology in *Perameles gunnii* have been documented in both Tasmania and Victoria (Barker *et al.*, 1975; Munday *et al.*, 1978; Obendorf and Munday, 1990). This documentation is by no means complete with many parasite species and their pathogenicity remaining unknown or their taxonomy

currently being under review (Norman and Beveridge, 1999). In the course of necropsy examinations of *P. gunnii*, numerous parasites have been recovered and identified and a checklist of parasites from *P. gunnii* has been drawn up by Obendorf and Munday, (1990). This list includes all known protozoan, helminth and arthropod parasites reported in *P. gunnii* from Tasmania and Victoria (Appendix 11.2 and 11.3).

During the present study, post mortem examinations were performed on *P. gunnii* found dead on road sides, killed by dogs or those which died from natural causes on private properties in southern Tasmania. Particular emphasis was placed on toxoplasmosis due to its devastating effects on the southern marsupial population. Whenever possible, material collected was screened to provide the serological status of *P. gunnii*. Fresh scats were collected during a three year capture and release program with Tasmania's Parks and Wildlife Services and additional faecal specimens were collected wherever possible and screened for parasites. In all, fifteen post-mortems were performed in a one year period but five animals had deteriorated badly when presented for post-mortem examination. In some instances the condition of the animal was too poor, with little if any material preserved, due to trauma and exposure to the elements and scavengers.

6.2 MATERIALS AND METHODS

6.2.1 Collection of specimens

Faecal specimens from bandicoots were collected on capture and release field trips at Huonville and Grove in southern Tasmania. Scats were collected from animals held in captivity. All faecal samples from the field were collected into wide-mouthed, water tight, sterile containers with tight-fitting lids. Some scats were collected into sterile screw cap containers and transported in Stuarts Transport medium. This medium was included as an option to perform bacteriological work that is discussed in Chapter 6, section B. Specimens brought back to the laboratory were promptly examined using the parasitological techniques described in Chapter 2; 2.7, or fixed in

10% formal buffered saline for later examination and refrigerated at 4°C until required.

Bandicoots found as road kills or dog kills were examined as soon as possible after collection. Bandicoots were weighed and sexed. Any tattoo numbers in ears were recorded and the location of previous capture and release was compared with the site where the animal had died. Before any dissection, ectoparasites including ticks, fleas and mites were collected and any skin abnormalities or signs of trauma noted. Animals were autopsied in a dorsal recumbency position as outlined by Lenghaus *et al.*, (1990). Organs were inspected before removal and any significant findings recorded. Gross pathological findings were reported and where possible histology was performed on all organs and tissues. Immunohistochemistry for detection of *T. gondii* was performed the procedure being described in the materials and methods chapter. Tissue imprints and smears were performed to screen for protozoal infections such as toxoplasmosis and sarcocytosis.

Swabs from sites relating to suspected infectious disease were cultured on horse blood agar at 37°C and significant bacteria identified using standard bacteriological techniques. Sampling of intestinal contents was obtained by sterile dissection during autopsies. The contents were examined with a dissecting microscope before processing by parasitological techniques. Representative numbers of any worms found were preserved in 90% ethyl alcohol with 5% glycerol.

6.2.2 Parasitological Procedures

Specimens were examined for the presence of protozoan trophozoites and cysts and helminth eggs and larvae using direct wet mount and a combination of formalin-ethylacetate sedimentation concentration (Garcia and Bruckner, 1993), using Johns Parafilter System (Biolab Scientific Pty. Ltd), and Zinc Sulphate Flotation Method (Smith and Bartlett, 1991). Where possible, fresh stool smears were made and

stained with Trichome stain. All helminth eggs recovered were measured using an eyepiece micrometer. Examination of repeat faecal samples was thought to be a reliable method of detecting intestinal parasites but was not always possible to obtain repeat samples from individual native animals, particularly in a capture and release investigation.

6.3 RESULTS

6.3.1 Fresh Scats

In the course of the investigation, approximately eighty-four faecal specimens were screened for parasites. The total parasite burdens were not recorded during this study, but the sparsity of parasites from specimens detected suggests the burden in *P. gunnii* was invariably low. The prevalence of adult worms was not great and the variety of species was limited. Parasitological methods performed on fresh scats has detected the first record of *Trichuris* spp., in *P. gunnii,* (Figures 6.1 and 6.2), *Eimeria* spp., (Figure 6.3), strongyle larva and eggs (Figures 6.4 a-e), *Giardia* sp., (Figure 6.5). The *Trichuris* eggs measured 65-70 μM in length. *Eimeria* oocysts measured approximately 20 μM. During this investigation *Giardia* species were reported and further work was expanded in this area and the results are discussed in Chapter 5.

6.3.2 Autopsy

Five bandicoots collected as road kills were considered to be in poor condition. Post-mortem examination revealed tissues were moderately to severely autolysed in these animals and therefore, could not be used for histological examination. Gross examination revealed nothing more than trauma in all but one case. Parasite load was not significant and in some instances specimens were poorly preserved due to the time lapse between death of the animal and fixing of faecal specimens. In one of the five bandicoots, one female at post-mortem examination revealed congested lungs with small haemorrhages. The liver was slightly congested with pale mottling seen on the surface to extend throughout the organ. Small haemorrhages or petechiation

Figure 6.1

Trichuris sp., in a faecal concentrate from *P.gunnii*. Approx 65 μm in length.

Stain: Iodine
Bar = 25μM.

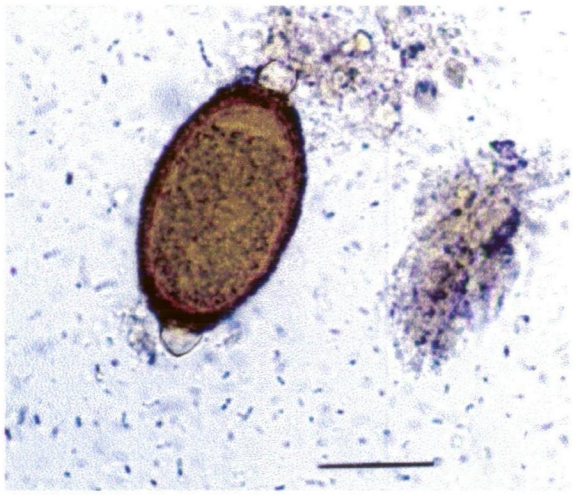


Figure 6.2

Trichuris sp., approx. 70 μm in length.

Stain: Iodine
Bar = 50 μM

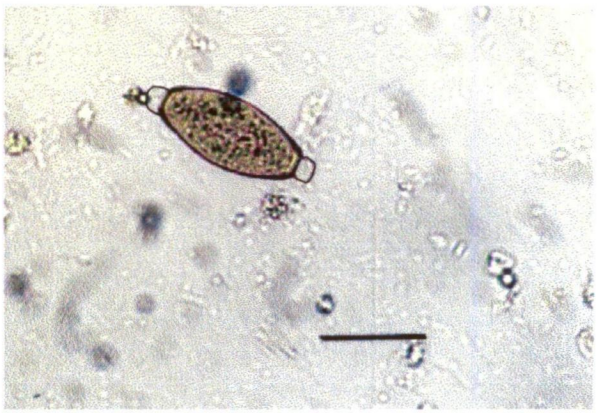


Figure 6.3

Eimeria sp., from *P. gunnii*.

Stain: Iodine
Bar = 20 μM

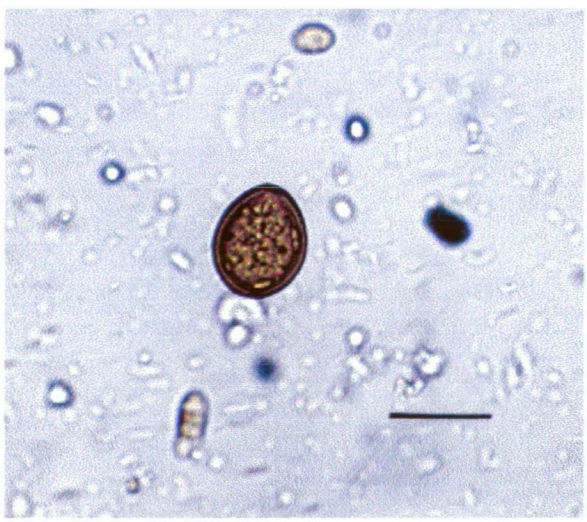


Figure 6.4 (a)

Immature strongyle egg in a faecal concentrate from *P. gunnii*.

Stain: Iodine
Bar = 25 μM.

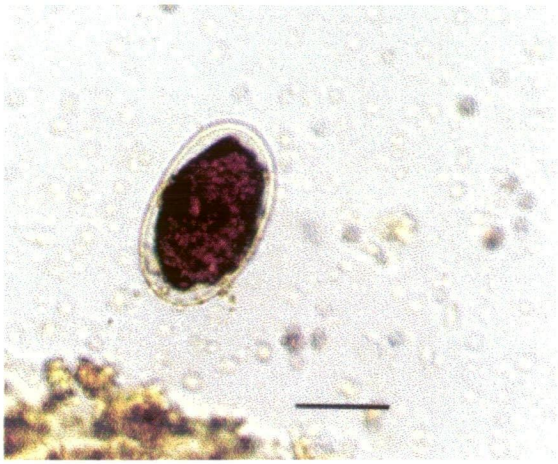


Figure 6.4 (b)

Developing strongyle egg, larva in a figure of eight shape.

Stain: Iodine
Bar = 25 μm.

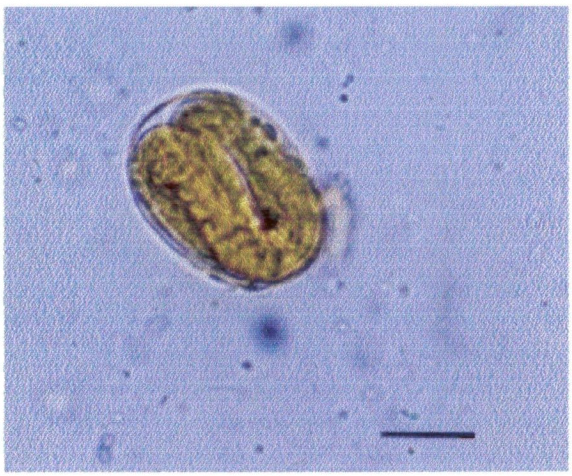


Figure 6.4 (c)

Hatching strongyle egg, larva in a figure of eight shape.

Stain: Iodine
Bar = 25 μM.

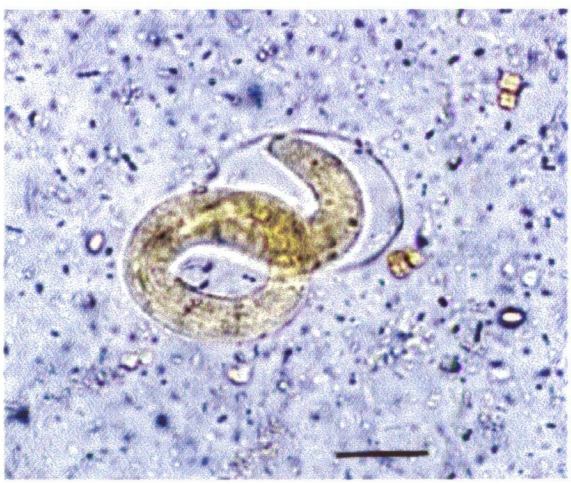


Figure 6.4 (d)

Strongyle larva in a faecal concentrate from *P. gunnii*. Approx. 400 - 500 μM in length.

Bar = 50 μM.

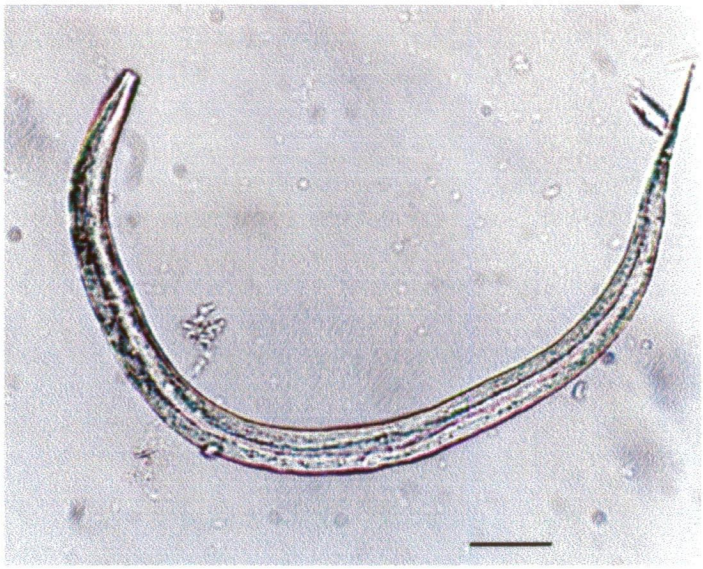
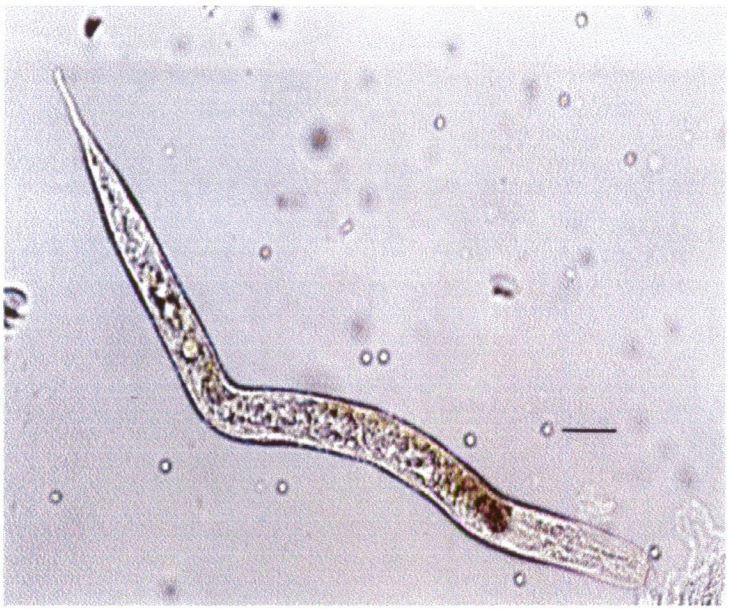


Figure 6.4 (e)

Strongyle larva in a faecal concentrate from *P. gunnii*. Approx. 400 - 500 μM in length.

Bar = 50 μM.



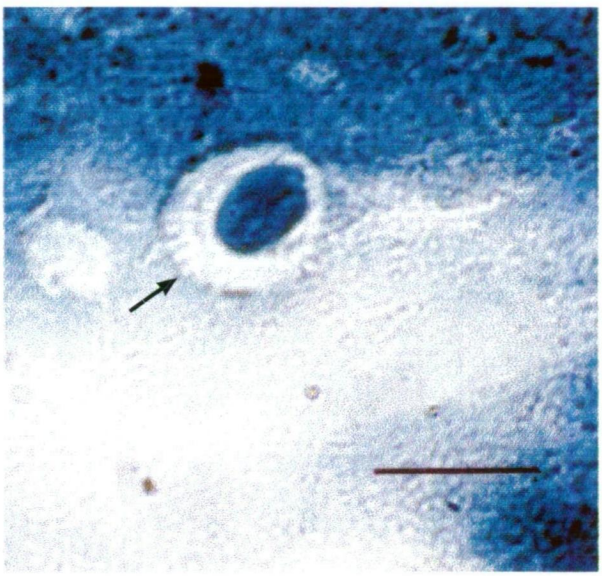


Figure 6.5

Cyst of *Giardia* with cyst stained using Trichome stain. Note visible halo (arrow) resulting from cyst shrinkage during permanent staining.

Permanent stain: Trichome
Bar = 10 μM

were detected in the stomach and slight congestion was noted in some areas of the small intestine. The stomach was empty and bowel content examination revealed strongyle spp., possibly *Parastrongyloides australis* eggs and adults. *Trichuris* eggs and *Eimeria* oocysts were occasionally detected. Tissue impression of the liver and spleen did not reveal *T. gondii* tachyzoites or other protozoa. Results are summarised in Table 6.1. Overall, worm burden appeared low.

During the population study at Grove, three adult bandicoots died of trauma due to predation by dogs. One animal was reported with positive serology for *T. gondii* although no gross lesions were visible at post-mortem. Tissue imprints did not reveal tachyzoites and none were detected by histology or immunohistochemistry. An acanthocephalan, *Australiformis* sp., was detected attached to the stomach tissue in one animal (Figure 6.6). Again these animals did not appear to carry an excessive worm load, worms being scanty in number and only a small range of species being found in each bandicoot.

One animal reported as a fresh road kill, was collected near the grid site at Huonville. This animal was tattooed and had been part of an ecological population study carried out by Tasmania's Parks and Wildlife Service over a two year period. On close examination this animal had a tooth abscess and was suffering from oral candidiasis. Gross lesions detected included petechiae of the small and large intestine. Lungs and heart showed severe haemorrhage with a blood clot noted in the left aorta. This may have been as a result of the traumatic impact at death or the possibility of general toxaemia or septicaemia. The male adult could not be aged. Stomach and small intestine were empty revealing it had not eaten for some time and was suffering from dehydration. Histology did not reveal *T. gondii* and tachyzoites were not detected in the tissue imprints. No tissue cysts resembling sarcocysts were seen in the muscle tissue of any of the animals.

Animal	Postmortem Pathology	Histological Findings	*T. gondii* Serology & Tissue Impression	Parasites
Road Kill (F)	**A, B,C,D,H**	Autolysed	Neg	Strongyle *Trichuris* sp.
Road Kill (F)	**H**	Autolysed	Neg	Strongyle sp.
Road Kill (M)	**H**	Autolysed	Neg	Strongyle
Road Kill (M)	**H**	Autolysed	Neg	Strongyle *Trichuris* sp.
Road Kill (M)	**A, D, H**	Autolysed	Neg	Strongyle *Trichuris* sp.
Road Kill (M)	**H**		Pos	Strongyle sp.
Road Kill (F)	**H**		Neg	*Eimeria* sp.
Road Kill (F)	**H**		Neg	Strongyle.
Road Kill (F)	**H**		Neg	Strongyle
Road Kill (M)	**H**		Neg	*Trichuris* sp.
Road Kill (M)	**H**		Neg	*Trichuris* sp.
Dog Kill (M)	**H**		Neg	*Australiformis* sp.
Dog Kill (M)	**H**		Neg	Strongyle
Dog Kill (F)	**H**		Neg	Strongyle
Road Kill (M) Tattooed Parks & Wildlife Service Program	Oral candidiasis White exudate Loose teeth Dehydration Anorexic Lung and heart **H**		Neg	Strongyle

Table 6.1 Clinical, serological, parasitological and postmortem findings in *P. gunnii*

KEY: **A** = Congestion & oedema of lungs, **B**= Necrosis & haemorrhages in liver, **C** = Stomach was empty, **D** = Petechiation of stomach and small intestine, **E**= Petechiation of large intestine, **MNL** = Mesenteric lymph node; enlarged, mottled and haemorrhagic, **H** = Haemorrhage due to trauma.

Figure 6.6

Proboscis showing rows of hooklets of the acanthocephalan, *Australiformis* sp., detected in *P. gunnii*

Bar = 20 μM

The six remaining animals did not reveal any significant gross pathology or histopathology. Faecal specimens of the stomach, small intestine and large bowel revealed a low parasitic burden.

6.3.3 Ectoparasites

Fleas and ticks presented only on fresh kills, or they were associated with wrappings used to transport the bandicoot to the laboratory. No mites or lice were found on any animals screened. Gross evidence of 'blood meals' left by fleas was evident in only four animals.

6.4 DISCUSSION

Overall most parasites identified from *P. gunnii* scats and gastric content at postmortem were similar to those reported by Obendorf and Munday, (1990) and the checklist adapted from Spratt *et al.*, (1991). All helminth species collected in this study have been reported previously from *P. gunnii* in Tasmania except for a *Trichuris* sp., belonging to the superfamily Trichuroidea, although, *Trichuris perameles* has been identified in the *P. nasuta* (Long-Nosed Bandicoot), *I. macrourus* (Northern Brown Bandicoot) and *I. obesulus* (Southern Brown Bandicoot) (Spratt *et al.*, 1991). It was initially thought the eggs of this species belonged to *Capillaria* spp., which have been recorded from *P. gunnii*, but the fact that the polar plugs protrude, however, led to the presumptive identification of *Trichuris* sp. Without the adult worm the species of *Trichuris* from *P. gunnii* cannot be established. The zoonotic potential of this species is also unknown.

The two most commonly reported *Trichuris* species from animals are those belonging to man and dog. *Trichuris trichiura*, as the human whipworm, is cosmopolitan in distribution but is more common in warm, moist regions (Beaver *et al.*, 1984). This species has been reported in other primates and pigs. *Trichuris vulpis* occurs in the caecum and other parts of the intestine in the dog and fox. *Trichuris ovis* has been

recorded from the caecum of the goat, sheep, cattle and many other ruminants while *T. globulosa* is the common form found in ruminants in South Africa and possibly other parts of the world. Beveridge and Green, (1981) have reported species of *Trichuris* in Australian domestic ruminants. *Trichuris* spp., have been isolated from cats in Australia including *Trichuris campanula* von Linstow, 1889, (Ng and Kelly, 1975) and *T. serrata* von Linstow, 1879 (Holmes and Kelly, 1972; Kelly, 1973).

In this investigation a worm belonging to the Phylum Acanthocephala was identified in the stomach contents of one autopsy case. Three species of Acanthocephala have been recorded in *P. gunnii*: *Australiformis semoni* (Linstow, 1898), *Plagiorhynchus* (*Prosthorhynchus*) *cylindraceus* (Goeze, 1782) Schmidt and Kuntz, 1966, (Smales, 1988). *A. semoni* is a common adult acanthocephalan of marsupials reported in Peramelidae and Dasyuridae from Australia and New Guinea (Schmidt and Edmonds, 1989). Its singularity, and the fact it was formerly placed in the genus *Moniliformis*, is often suggested by authors, in the absence of alternative hypotheses, as suggesting an origin from rodents (Beveridge and Spratt, 1996). Reports of *Australiformis/Moniliformis semoni* usually identify the adult parasite as being deeply embedded in the mucosa (Lenghaus *et al.*, 1990). *Plagiorhynchus* (*Prosthorhynchus*) *cylindraceus* (Goeze, 1782) Schmidt and Kuntz, 1966, has been recovered from the peritoneum of the bandicoot *Isoodon obesulus*, and the intestine of *P. gunnii* which was the first reported case in a mammal (Smales, 1988). *Plagiorhynchus* (*Prosthorhynchus*) *cylindraceus* was usually considered a parasite of passerine birds until described by Smales, (1988). Different species of Acanthocephala and multiple infections have been reported to cause local trauma and inflammation at the site of attachment to the intestinal wall and often perforation, in both humans and animal species (Neafie and Marty, 1993; Beaver *et al.*, 1984). In many species, the parasites can produce anaemia, weight loss and weakness (Lapage, 1962). The only acanthocephalan recorded from humans in Tasmania is one case of infection with

Moniliformis moniliformis imported to Hobart in a young child from Darwin (Bettiol and Goldsmid, *in press*).

Numerous nematodes have been described in *P. gunnii* from Tasmania and Victoria. These include *Peramelistrongylus australis* Mawson, 1960, *Peramelistrongylus skedastos, Labiobulura inglisi, Physaloptera* sp. *Echinonema cinctum, Echinonema* sp. n., *Parastrongyloides australis. Capillaria* sp. (undescribed), *Marsuptostrongylus bronchialis, Cercopithifilaria johnstoni* (Appendix 11.3).

Capillaria spp., have been sieved from stomach and intestinal contents of *P. gunnii* or histologically isolated from the tongue and oesophagus (Lenghaus *et al.*, 1990). Verminous bronchitis and bronchiolitis in marsupials is usually associated with *Capillaria* sp., that reside in the epithelial lining of bronchi and bronchioles (Love and Reddacliff, 1992). In this investigation *Capillaria* spp., were not identified in any of the animals screened. In Australia, the parasite has been reported in dogs and cats (Stevenson and Hughes, 1988) and in a survey of feral cats in Tasmania, Milstein, (1993) recovered unidentified *Capillaria* spp., eggs from 4.8% of these animals raising the possibility that native animals become infected through feral cats. It is reported that the pulmonary *Capillaria* sp. from cats can at times infect humans (Stevenson and Hughes, 1988).

Peramelistrongylus spp., is a gastric parasite commonly found in members of the marsupial families Dasyuridae and Peramelidae (Durrette-Desset and Beveridge, 1981), but it was not found in this study of *P. gunnii.* Numerous reports of *Strongyloides/Parastrongyloides australis* have been reported from post-mortems of *P. gunnii* (Lenghaus *et al.*, 1990). Larval nematodes have been identified embedded in the mucosa of the small intestine, which can lead to fluid and electrolyte loss. Infections of these nematodes along with *Capillaria* spp., *A. semoni,* and *Physaloptera* sp. were often identified in animals suffering from toxoplasmosis by

Lenghaus *et al.*, (1990). The life cycle of these nematodes is unknown, but *Parastrongyloides* larvae are known to penetrate the skin, entering the vascular system to pass to the lungs in a similar way to other *Strongyloides* species. Larval nematodes have been located in the alveoli of *P. gunnii*, where they move up the trachea to be swallowed (Lenghaus *et al*, 1990). In the intestines the larvae mature to adult forms and the characteristic thin-shelled eggs containing first-stage larvae are passed in the faeces. No evidence has been collected in regards to cross-transmission but Smales and Mawson, (1978) surveyed worm burdens in feral goats, possums and Kangaroo Island Wallabies from places where macropods and ungulates graze attempted to together. They also experimentally infected sheep and rabbits with *Labiostrongylus eugenii*, a strongyle of the Kangaroo Island Wallaby. Neither of these investigations gave any evidence to suggest that cross-transmission of strongyles between macropods and domestic stock occurred (Smales and Mawson, 1978) and the possibility of these marsupial helminths being zoonotic has not been studied.

Two genera of filarioid nematodes are present in bandicoots, *Breinlia* and *Cercopithifilaria* but only one has been identified in *P. gunnii, Cercopithifilaria johnstoni.* This species has been reported in the subcutaneous connective tissues in a range of eutherian and marsupial hosts in eastern Australia (Mackerras, 1954, 1962; Spratt and Varughese, 1975). These dermal microfilariae have been identified in *P. gunnii* and in northern Australian bandicoots *Perameles nasuta* and *Isodoon macrourus*. Vuong *et al.*, (1993) identified lesions in animals infected all with pathology caused by the microfilariae exiting the lymphatic vessels, giving rise to a localised inflammatory reaction leading to fibrosis. Spratt and Haycock (1988) made a comprehensive study in the aspects of the life history by experimentally infecting the species into a wild population of bush rats (*Rattus fuscipes*) and laboratory rat (*R.. norvegicus*) revealing this filaroid nematode induces skin and eye lesions in all infected animals. The life cycle was elucidated by Spratt and Haycock, (1988) in ticks belonging to the genus *Ixodes*.

Adult nematodes of the genus *Physaloptera* Ruldophi, 1819, were reported to occur in a variety of avian and mammalian hosts including Australian marsupials (Spratt *et al.*, 1991; Norman and Chilton, 1994). Insects are considered intermediate hosts (Hobmaier, 1941; Gray and Anderson, 1982) with reptiles as paratenic hosts (Widmer, 1970). Four species have been described from the stomachs of bandicoots and bilbies in central and eastern Australia, including *P. gunnii* and *I. obesulus*. These species are capable of burrowing causing stomach and intestinal damage.

Recently the species of *Physaloptera* Ruldophi occurring in the bandicoots (Families Peramelidae and Thalacomyidae) of Australia were re-examined and re-described (Norman and Beveridge, 1999). Norman and Chilton, (1994) revealed fixed genetic differences between *Physaloptera* Ruldophi isolated in *P. gunnii* from Victoria and *P. nasuta* from Queensland. This suggested that each host was infected by a different species of *Physaloptera* Ruldophi leading to the possibility that the nematodes found in Tasmanian *P. gunnii* differs from those found in the Victorian *P. gunnii*. In addition the current morphological study shows that the specimens from *P. gunnii* differ in their arrangements of pedunculate papillae (Norman and Beveridge, 1999). Some species of *Physaloptera* from animals have been reported to infect humans (Goldsmid, 1968), but whether those from marsupials are zoonotic is not known.

Overall trematodes appear to be an uncommon parasite in Macropodoidea with only three species reported in free-ranging hosts. Within *P. gunnii* only one, *Mehlisia acuminata* Johnston, 1913, has been reported from the small intestine, (Spratt *et al.*, 1991; Obendorf and Munday, 1990).

Of the cestodes only *Hymenolepis peramelidarum* Nybelin, 1917, has been recorded in *P. gunnii* (Spratt *et al.*, 1991). The life cycle of this species of cestode requires

intermediate hosts, usually invertebrates, although a species of man and rodents, *Hymenolepis nana*, has a direct or indirect life cycle (Beaver *et al.*, 1984).

Intestinal coccidiosis caused by the protozoan *Eimeria* sp., is a common cause of death in macropodids, particularly those held in captivity (Munday, 1988). *Eimeria* sp., has been detected in *P. gunnii* in Tasmania although original reports were from *P. gunnii* of Victoria. In the present report, intestinal coccidia were common and appeared to be nonpathogenic but Lenghaus *et al.*, (1990) indicated that their presence would likely be seen as an outbreak of diarrhoea, dysentery and death in animals kept under crowded conditions.

In this current investigation species of the sporozoon genus *Klossiella* sp., were not identified from current autopsy material. *Klossiella* sp. is considered not to be pathogenic and has been identified in both species of bandicoots from Tasmania (Barker *et al.*, 1975). Their morphology and development was closely related and proposed to be the same species, *K. quimrensis* (Barker *et al.*, 1975). This genus commonly parasitises the tubular epithelial cells of the renal cortex. Stages from gametocytes to mature sporocysts are often seen in parasitophorous vacuoles in cells of the thin loops of Henle, especially at the papillary border of the medulla. Apart from enlargement of the parasitised cell it appears to cause no damage (Speare *et al.*, 1984).

Though individual worm counts and geographic variables were not strictly evaluated in the present investigation, it is well known that parasites occupy specific and predictable sites in the intestinal tract of their host (Moore and Simberloff, 1990; Cislo and Caira, 1993). The low burden categorised in individuals may be a reflection of unfavourable conditions for helminths and other protozoa apart from *T. gondii*. However, *P. gunnii* are solitary animals so they would not normally tend to become overburdened with intestinal helminths, due to lack of reinfection. High

worm burden and consequent clinical disease would thus tend to reflect overcrowding. The season may influence the abundance of intermediate hosts for an intestinal helminth. In turn this can affect the prevalence and intensity of infection in the host (Martin and Huffman, 1980; Anderson and Skorping, 1991). The three year capture and release program was performed over a particularly dry period in Tasmania. No attempt was made in this present study to investigate any effect of season on nematode burden due to the lack of information on aspects such as seasonal changes in larva counts in *P. gunnii* habitat. Insect counts and food source for the bandicoot were in decline during the population study of 1993-1996 (Mallick *et al.*, 1997b). The drier conditions over the period were more patchy in both space and time (Bureau of Meteorology, unpublished data). Overall, however, the helminth burden recorded in the present investigation would suggest they had little effect on the general well-being of the animals studied.

Data of ectoparasite burden was collected and collated during the capture and release program / population study of *P. gunnii* with Parks and Wildlife Services. *P. gunnii* were found to carry ticks on the ears and fleas throughout the body. The degree of infestation of fleas was rated as medium, and the mean number of ticks on the ears of male and female averaged at about 0.75 per animal. Flea numbers varied between populations of animals but this variability was not evident with ticks. Male *P. gunnii* were more often infested with fleas than females (Mallick *et al.*, 1997b).

During post-mortem examination very few fleas and ticks were identified, due to the ectoparasites, especially the flea, leaving the host as the body cooled. Three fresh kills had one or two ticks on their ears and medium flea loads. Lenghaus *et al.*, (1990), reported that ectoparasites seemingly migrated from their host within hours of death. Several species of fleas have been identified on *P. gunnii*, including *Pygiopsylla hoplia*, *Pygiopsylea zethi*, and *Stephanocircus dasyuri*. There are no reports of their health significance to this marsupial but there is a strong possibility

that they may be the vectors of the blood-borne parasite *Trypanosoma* spp., identified during this investigation.

Ixodid ticks were identified in *P. gunnii*, the most common species being *Ixodes tasmanii*. *I. cornuatus* has been identified but no reports to date have identified tick paralysis in *P. gunnii* (McManus, personal communication). The tick remains a suspect vector of the blood-borne parasite *Hepatozoon*, identified in this study and discussed in Chapter 4.

Collectively the parasites detected and the diseases or pathological conditions reported are not necessarily peculiar to *P. gunnii*, but they may have a bearing on future management plans particularly in regard to captive maintenance or breeding. Where animals may be kept under conditions of stress and overcrowding so promoting transmission, higher worm burdens can develop and more likely clinical disease. In free ranging *P. gunnii* numerous deaths labelled as road kills or cat kills have been presented for examination and proved to have serious pre-existing diseases (especially toxoplasmosis) and which can lead to debility or disorientation, so they readily become victims of cars or predatory animals (Lenghaus *et al.*, 1990). With the exception of toxoplasmosis and *Giardia duodenalis*, the zoonotic potential of the parasitic infection of *P. gunnii* is unknown. No animals have been found infected with *Trichinella pseudospiralis* but again as *P. gunnii* is not used for human food (as are wallabies and possums) there is no real hazard for humans (Henry, 1989; Obendorf *et al.*, 1990). Of the ectoparasites, *I. cornuatis* is commonly found on humans while *T. tasmanii* is sometimes found.

B. Bacterial Isolates of Free-ranging *P. gunnii* with Special Reference to salmonellae in Tasmanian Wildlife.

6.5 INTRODUCTION

Diseases caused by bacteria of zoonotic potential have been described in native animals and domestic animals throughout Australia (Stevenson and Hughes, 1988; Hough, 1992). One most commonly associated with diarrhoea and enteritis in the human population is *Campylobacter jejuni* and less commonly *Campylobacter coli.* Reports on the source of infections have included dogs, cats, domestic birds and marsupials (Hough, 1992). Animals often present with the same gastroenteric problems as humans but in many instances can be asymptomatic (Hough, 1992). Notification rates of *Campylobacter* spp., in Tasmania are relatively high and are the most common cause of gastroenteritis in the State followed by *Salmonella* spp., especially *S.* Typhimurium and *S.* Mississippi (Anon, 1999).

Salmonellae are well documented amongst warm and cold-blooded animals throughout the world. In Australia, there is a wide range of salmonellae present in the environment with regional differences and differences in the prevalence of serovars. In Tasmania, human salmonellosis is mainly due to *Salmonella* Typhimurium and *Salmonella* Mississippi with increasing numbers of human and animal infections due to *S.* Mississippi. It is commonly observed that while human infections with *S.* Mississippi are rarely encountered on mainland Australia, the National Salmonella Surveillance Scheme (NSSS) reports 96% of all such *S.* Mississippi infections notified in Australia can be traced back to Tasmania (Anon; 1991). *S.* Mississippi has been isolated from a range of domestic animals, including, cats, dogs and goats as well as native species such as bandicoots, wombats, pademelons, wallabies, possums, Tasmanian Devils, quolls, ravens, tiger snakes, lizards and skinks (Anon; 1990). Davies (1995a) found that Tasmania's

native animals comprise a significant potential reservoir for a wide variety of *Salmonella* spp.

The first reports of *Salmonella* spp. from bandicoots were by Lee and Mackerras, (1955). Two *Salmonella* spp were identified in animals that had died in captivity, while *Salmonella* Lexington was isolated from *Isoodon obesulus* (short-nosed bandicoot) and *S.* Newport was isolated some time after capture from a bandicoot that was negative on primary examination. In both cases these infections were considered to be acquired infections. More recently Ball, (1991) reported *S.* Mississipi in *P. gunnii* and *I. obesulus* from Tasmania with serovar *S.* Warragul being detected in *I. obesulus.*

Multiple infections of *Salmonella* and *Arizona* have been recorded from wild marsupials with variable rates of detection (Marx, 1969, Iveson and Bradshaw 1973, Kourany *et al.*, 1976). These rates ranged from 12 to 71% amongst marsupials, with rates of infection reported to be as high as 20 to 100% amongst wild reptiles.

The digestive tract of the bandicoot appears to be more adaptable to diets of different qualities, allowing the bandicoot to switch between insect and plant foods, and thus to exploit nutritionally variable environments (Hume and Moyle, 1995). As a terrestrial marsupial and small hindgut fermenters, bandicoots are considered to have a less differentiated digestive tract than any of the arboreal folivorous marsupials (Hume and Moyle, 1995), but the caecum and proximal colon are regions with significant microbial activity, and a possible explanation for the relatively high digestibility of plant fibre reported by Moyle *et al.*, (1995). Digging for earthworms and coprophagic insects, however, may expose *P. gunnii* to infectious agents such as *Salmonella* and other pathogenic bacteria not only from eating insects but ingesting soil and organic matter while foraging for food. Though the bandicoot is considered an omnivore feeding on insects as well as eating grass and fruit, they have been noted to be occasional opportunistic carnivores,

scavenging from carcasses when available (Goldsmid, personal communication). This behaviour is reflected in other animals such as the native cat that have been identified to be infected with *S.* Mississippi and excluding any other serovar (Ball, 1991). Humans can then be infected through bacteria in soil, plants and in water.

6.6 MATERIALS AND METHODS

6.6.1 Collection of Specimens

Specimens from bandicoots were collected on field trips across the south and south-west of Tasmania. Scats were collected from animals held in captivity. All scats were collected into sterile screw cap containers and transported in Stuarts Transport medium. Sampling of intestinal contents was obtained by sterile dissection during autopsies and the use of sterile swabs to obtain specimens. Organs such as lung, liver and spleen were cultured. Upon arrival at the laboratory, specimens were examined for potential enteric bacterial pathogens by direct plating of both unenriched and enriched samples.

6.6.2 Processing of Samples

Specimens were directly plated for examination of potential enteric bacterial pathogens onto media selective for enteric organisms such as MacConkey Agar (CM7; Oxoid, NSW, Australia), Xylose-Lysine-Desoxycholate Medium (XLD Medium; CM460, Oxoid NSW, Australia), and Hektoen Enteric Agar (HEK, CM410, Oxoid, NSW, Australia). All specimens were plated onto Blood Agar (Tasmanian Laboratory Services, Tasmania, Australia) for a non-specific examination of aerobic growth. Plates were incubated overnight at 37°C. The intestinal and faecal samples were assessed for anaerobic growth on Blood Agar under anaerobic incubation systems.

The methodology used in the isolation of *Salmonella* was dependent on the specimen tested. Collected specimens and swabs were resuscitated in two broths,

buffered peptone water (CM509B, Oxoid, NSW, Australia) and Tryptone Soya Broth (TSB; CM129, Oxoid, NSW, Australia). If the specimens were found to be well formed, the faeces were made into a paste with a small amount of sterile distilled water to homogenise the sample before adding to broth. All broths were incubated overnight at 37°C. After incubation, a 3 mm loop of enrichment culture was transferred onto the aforementioned media for further screening and incubated overnight at 37°C. All plates were examined for possible enteric pathogens, and for general commensals.

One mL of incubated resuscitation broth was added to Selenite Broth (CM399 and L121 Oxoid, NSW, Australia) and 0.1 mL added to Rappaports RV Broth (CM669B, Oxoid, NSW, Australia) as a selective enrichment step. Broths were incubated for 18-24 hours at 37°C. After incubation, a 3 mm loop of enrichment culture was transferred onto the aforementioned media for further screening and incubated overnight at 37°C. All plates were examined for possible enteric pathogens, and for general commensals.

MacConkey medium was selected for its suitability for the cultivation of pathogens that may be present in a variety of specimens, and for its ability to differentiate between lactose-fermentors (such as typical *Escherichia coli*) and non-lactose fermenting organisms (such as *Salmonella*). Both HEK and XLD can support a diverse range of enterics, including *Escherichia coli.* Blood agar was utilized due to its good non-selective general purpose medium for recovery of aerobic and anaerobic organisms.

6.6.3 Identification of Isolates

The isolates obtained from faecal specimens collected during field work and autopsies were examined. Organisms considered to be normal commensals were identified by virtue of their colonial characteristics, gram morphology and by the oxidase test. Suspect colonies were identified biochemically.

Biochemical identification was carried out with the Microbact 24 E system (MB152W; Medvet Science, Oxoid, NSW, Australia) used to identify gram negative bacilli and Microbact 24 AN for anaerobic isolates. The Analytical Profile Index (API) 20E package (bioMerieux sa, Marcy l'Etoile, France) was utilised often as a supplementary test to confirm the identification of strains that gave uncertain results with the Microbact system.

The Microbact 24E and 24 AN system utilises a conventional 96 well microtitre plate, with two rows of biochemical substrates (24 wells) allocated for identification of each isolate. Strains were inoculated into the kit according to the manufacturer's directions, incubated at 37°C overnight, and read after the addition of specified reagents (MB601, Oxoid, NSW, Australia). Each well was scored '+' or '-' using the colour chart provided with the kit, then entered into the Microbact 24 E database package for the analysis of the data and calculation of the most probable organism using an octal code. The API 20E system comprises a series of cupules containing freeze-dried biochemical substrates. Each cupule was inoculated with the isolate according to the manufacturers directions and incubated at 37°C overnight. The cupules were read after the addition of specified reagents and analysis of the data performed with API database.

6.7 RESULTS

Twenty-five specimens collected from field work and captive *P. gunnii*, and intestinal contents from six autopsies were screened for enteric pathogens using a

Animal	Bacterial Species Isolated from *P. gunnii*						
No. 1	*E. coli*	*P/M morganii*				*Arizona*	
No. 2	*E. coli*	*P/M morganii*				*Arizona*	
No. 3	*E. coli*		*S. marc.*	*C. freundii*	*E. aggl.*		
No. 4	*E. coli*	*P. stuartii*	*S. marc.*				
No. 5	*E. coli* inactive	*P. mirabilis*		*C. freundii*		*Salmonella spp ?*	
No. 6	*E. coli*	*P/M morganii*	*S. lique.*	*C. freundii*		*Salmonella spp ?*	
No. 7	*E. coli*	*P/M morganii*	*S. lique.*				
No. 8	*E. coli*	*P/M morganii*		*C. freundii*	*E. aggl.*		*Clost. spp.*
No. 9		*P/M morganii*		*C. freundii*			
No. 10	*E. coli* inactive				*E. aggl.*		*Clost. spp.*
No. 11	*E. coli* inactive	*P/M morganii*	*S. lique.*				*Clost. spp.*
No. 12	*E. coli*			*C. freundii*			
No. 13	*E. coli* inactive		*S. lique.*		*E. aggl.*		*Clost. spp.*
No. 14	*E. coli* inactive	*P/M morganii*				*H. alvei*	
No. 15	*E. coli*		*S. lique.*				
No. 16	*E. coli*						*Clost. spp.*
No. 17	*E. coli*	*P. mirabilis*		*C. freundii*			
No. 18	*E. coli*		*S. lique.*		*E. aggl.*		
No. 19	*E. coli* inactive	*P/M morganii*	*S. lique.*			*H. alvei*	
No. 20	*E. coli*			*C. freundii*			
No. 21	*E. coli*						
No. 22	*E. coli*				*E. gerg.*		
No. 23		*P/M morganii*					*Clost. spp.*
No. 24	*E. coli*		*S. lique.*			*K. ozaenae*	
No. 25	*E. coli*	*P/M morganii*					
PM 1	*E. coli*		*S. lique.*			*K. ozaenae*	*Clost. spp.*
PM 2	*E. coli*			*C. freundii*			*Clost. spp.*
PM 3	*E. coli*	*P/M morganii*					*Clost. spp.*
PM 4	*E. coli*	*P/M morganii*			*E. aggl.*		*Clost. spp.*
PM 5	*E. coli*	*P/M morganii*		*C. freundii*			*Clost. spp..*
PM 6	*E. coli*			*C. freundii*		*K. ozaenae*	*Clost. spp.*

Table 6.2 List of bacterial isolates detected from wild *P. gunnii* faecal specimens and at post mortem.

KEY: *E. aggl* = *E. agglomerans*, *E. gerg.* = *E. gergoviae*, *Clost.* spp. = *Clostridium* spp, *S. marc.* = *S. marcescens* , *S. lique.* = *S. liquefasciens*, PM – postmortem

variety of solid agars and broths. Table 6.2 provides an indication of key organisms identified. *E. coli* was the most common isolate identified and detected in faecal specimens. *Proteus* spp. was isolated from 14 scats and included *Proteus mirabilis*, *P. stuartii*, and *P. morganii/Morganella morganii*. *Serratia liquefasciens*, *S. marcescens*, *Citrobacter freundii*, *Enterobacter agglomerans*, *E. gergoviae*, *Hafnia alvei*, *Klebsiella ozaenae* and *Arizona* sp., were commonly identified. The two suspect *Salmonella* species were identified with the biochemical test kits but were serotyped at a reference laboratory (IMVS, Adelaide, South Australia) as non-*Salmonella* serovars. *Proteus* spp. and *Enterobacter agglomerans* were the most common isolates grown from tissues samples. Anaerobes were identified in only six scats but were grown from all autopsy samples. *Clostridium* spp. was most common isolate.

6.8 DISCUSSION

The bacteria detected from scats and gut contents of *P. gunnii* in many instances were considered commensals of these animals belonging to the family Enterobacteriaceae. At an early stage some balance is struck between the young marsupial and its microbial flora, yet the interrelationship is a complex one. Early work by Smith and Crabb, (1961) and Smith, (1965), in a variety of young domestic animals suggested the first organisms to colonize the alimentary tract in most species include *Escherichia coli*, *Clostridium welchii* (= *C. perfringens*) and *Streptococcus* spp., which appear within a few hours of birth. Lactobacilli and other bacteria soon follow in particular species, while yeasts are not found in animals whose diet consists solely of milk (Yadav *et al.*, 1972b).

The most common isolates that were detected in faecal specimens of *P. gunnii* were *E. coli* and *Proteus* spp. The *Proteus* spp., *Morganella morganii*, and *Enterobacter* sp., are often detected and commonly isolated from autopsy specimens, including tissue samples. First suggestions would indicate that these microorganisms are part

of the soil intake that normally occurs with bandicoots foraging for food as most isolates were considered opportunistic pathogens found in soil and water. There have, however, been reports of septicaemia in *P. gunnii* due to these organisms, which have resulted in death. Septicaemia has been recorded in animals following injury to the toes, injury while in captivity and in one case, associated with a trapping injury (Booth and McCracken, 1995). The organisms recorded as the cause of septicaemia were *Morganella morganii*, *Vibrio metschnikovii*, *Staphylococcus aureus*, *E. coli* and *Proteus* spp. Septicaemia unrelated to injury was recorded in a male *P. gunnii* possibly affected by the stress of recent weaning and enclosure change. *E. coli* and *Proteus* spp. were cultured from this animal (Booth and McCracken, 1995). Other reports of severe exudative dermatitis, conjunctivitis and pouch infection have been reported in a *P. gunnii* within three months of capture and confinement to an enclosure. Opportunistic pathogens reported included *E. coli*, *Staphylococcus aureus*, plus *Corynebacterium sp.*, *Enterobacter sp.*, and *Streptococcus* spp., isolated at postmortem (Lenghaus *et al.*, 1990).

Several bacterial species were isolated in this investigation the clinical significance of which is unknown. *Serratia marcescens* is an opportunistic pathogen that is a common saprophytic microorganism found in soil and water but rarely in the intestinal tract of humans or animals (Boyd and Hoeri, 1986). *Serratia liquefasciens* has often been reported to cause a range of infections in humans including septicaemia, urinary tract infections and diarrhoea (Serruys-Schoutens *et al.*, 1984; Brooks *et al.*, 1988; Woodfield, 1991). The bacterial species has been reported as a pathogen causing severe illness and possibly death in young marsupial species such as the koala (Osawa *et al.*, 1992). *S. liquefasciens* has been reported as the cause of mastitis in domestic animals (Nicholls *et al*, 1981; Bowman *et al.*, 1986). *Hafnia alvei* has been reported as a causative agent of intestinal disorders and is found in different environments. Recently it has received increased clinical

attention, as a cause of diarrhoea and a range infections in humans (Rodriguez *et al.*, 1999).

The occasional detection of *Arizona* species is not surprising as it has often been detected in animal species, and considered pathogenic for snakes, turkeys, chickens and humans (Boyd and Hoeri, 1986). *Arizona* species resemble *Salmonella* species serologically and biochemically, but *Arizona* differs biochemically in that it shows delayed lactose fermentation and delayed gelatin hydrolysis and it does not ferment dulcitol. In this survey no confirmed *Salmonella* species were detected.

The incidence of disease in adult species may vary according to climatic season, as well as the quality of food intake by the animal. Investigations of the quokka, *Setonix brachyurus,* on Rottnest Island in Western Australia revealed a marked seasonal variation in the frequency of salmonellae isolated from the wild population (Iveson and Bradshaw, 1973; Hart *et al.*, 1985). An increase in the bacterial species occurred rapidly over several weeks and was believed by Hart *et al.*, (1985) to be due to the disruption of the digestive physiology of the marsupial caused by the decline of feed over the summer months. However, salmonellosis was not reported in *S. brachyurus.* Yet orphaned macropodids, joeys in particular, have a high prevalence of infection with salmonellae, causing gastroenteritis and septicaemia. In these cases promotion of infection by stress was strongly suggested (Speare and Thomas, 1988).

A survey by Ball, (1991) of Tasmanian native animals identified carnivorous animals as the main carriers of *Salmonella*, including the Tasmanian devil which is indigenous to Tasmania. Native cats were identified to be infected with *S.* Mississippi to the exclusion of any other serovar. These animals, like bandicoots, are more opportunistic carnivores, feeding on insects, especially corbie grubs. At the same time they have been noted to scavenge from carcasses when available as

well as eating grass and fruit. The common explanation for the predilection in native cats to one serovar was the bacterial/host affinity or a contaminated dietary component (Ball, 1991). With similar feeding habits, *P. gunnii* may become susceptible to a similar range of pathogens but its carcase scavenging habit is probably much less usual than in the native cat.

A high incidence of *Salmonella* in reptiles has been documented in several surveys in Australia (Iveson and Hart, 1983) while Ball, (1991) isolated *S.* Mississippi and *S.* Victoria from skinks, which are mainly insectivorous and detected different serovars from snakes and lizards. Reptilian representation in Tasmania is restricted to lizards or skinks and three species of snakes, which tend to prey on small, live creatures such as mice, birds, amphibians and insects. It was suggested by Ball, (1991) that native cats and skinks may be part of a small cycle involving particular serovars such as *S.* Mississippi. This raises the possibility of insects harbouring salmonellae that pass down the food chain and so may involve bandicoots. Josland, (1951) (cited by Ball, 1991), found that *Salmonella* can persist for several months in faeces, and have been recovered after several months in slurry (Best *et al.*, (1971) cited by Ball, 1991), sludge and soil (Thunegard, 1975) and in Tasmanian water ways (Ball, 1991). Scats from native cats, domestic stock, and other species (including humans) may attract an abundance of insects and earthworms, in turn attracting skinks and bandicoots.

The isolation methods for defining true rates of infection in wild populations is variable and differs amongst investigators (Hart *et al.*, 1982). In the present study a number of selective procedures were incorporated from past clinical and food investigations. Routine laboratory methods and enrichment techniques reported by Ball, (1991) were considered essential in resuscitating salmonellae from environmental sources, including scats. These techniques were implemented to provide the best recovery of all types of coliforms including *Salmonella* spp. The

more traditional medium such as XLD was the best option and has been proven to perform better than new chromogenic media that are currently available for isolation and presumptive identification of salmonellae (Pimbley and Gregory, 1997). With regard to other bacteria, Davies, (1995a) was unable to isolate as *Campylobacter* spp., from local native animals, and for this reason not attempted in this investigation. However, de Boer, (1994) (cited by Davies, 1995a) using the same culture techniques, carried out a study of water supplies and found that the organism could be isolated from water in rural farming areas with comparative ease. The fact de Boer, (1996) could not isolate *Campylobacter* spp. in any water supplies from national parks, appears to confirm the conclusions reached by Davies, (1995a) that native animals were not a source, and that domestic stock were probably responsible for the spread of this organism.

The bandicoot is noted as a potential reservoir for a number of bacterial zoonoses, and may contribute to human *Salmonella* infections in the state (Davies, 1995a; Ball, 1991). There is the possibility that human defaecation in wilderness areas and waste of domestic stock act as a possible source of infection. Even though no salmonellae were detected in *P. gunnii* from this study, potential pathogens were isolated. In many instances it is unknown what effect these strains may have on *P. gunnii* during times of nutritional deprivation, environmental stresses (e.g. severe winters, hot dry summers, captivity), injury, or concurrent disease. There are other instances when bandicoots have been implicated on mainland Australia as reservoirs of spirochaete and rickettsial diseases.

Bandicoots on mainland Australia have been implicated in reports of Lyme disease. The causative agent of Lyme disease is the spirochaete *Borrelia burgdorferi*, including other strains (Benenson, 1995). However, controversy remains surrounding the disease and the vector in Australia (Russell, 1998). Recent evidence suggests that the spirochaete may exist in New South Wales (Hudson *et*

al., 1998). Tick species known to carry the spirochaete in the northern hemisphere are not found in Australia, but reports suggest that other common *Ixodes holocyclus* or the bush tick are capable of acting as the vector. This tick has a complex epidemiology with a wide range of animal hosts, including native marsupials and domestic animals such as dogs and cattle, in addition to humans. Of the native marsupials the bandicoot is the most important animal in the epizootic cycles involving the bush tick (Munro, 1992).

In addition, another spirochaete *Leptospira*, has been reported in a wide range of hosts though clinical disease is uncommon. Humans are accidental hosts of this disease and may be infected if they come in contact with urine from infected rodents or larger animals (Faine, 1992). Prevalence of positive antibody titres to various serovars have been recorded in Tasmanian free-ranging Macropodidae including *Potorous tridactylus* (Munday, 1972b) and *M. rufogriseus rufogriseus* (Durfee and Presidente, 1979). Positive serology has been confirmed in the Long-nosed Bandicoot (*Perameles nasuta*) and Southern Brown Bandicoot (*Isoodon obesulus*) of south-eastern Australia (Milner *et al.*, 1981) but the serological status of *P. gunnii* for leptospire exposure in Tasmania is unknown and little is known of the effects on the bandicoot itself (McManus, personal communication).

Recent reports have also increased concern for tick-related effects and diseases in humans from many parts of Australia (Russell, 1998). These include reports of Tick typhus (Queensland Spotted Fever – *Rickettsia australis)*, along the east coast of Australia as far as Wilson's Promontory (southern Victoria), and Flinders Island Spotted Fever (FISF – *Rickettsia honei*) in Bass Strait and possibly northern Tasmania (Russell, 1998; Stewart, 1991). Reports of tick paralysis in children mainly due to ticks occur annually across the east coast of Australia. With local climate change, and warmer temperatures experienced during winter months the distribution of cases has increased further south.

Tick paralysis also affects domestic cattle, sheep, dogs and cats. Most cases of tick infestation in Tasmania occur during the summer and autumn months probably reflecting the activity of the adult ticks and their hosts. Recorded cases of animal paralysis are highest during this period, but as yet not recorded in humans. Other hosts include small macropods, wombats and a much wider range of native and feral animals (Green and Munday, 1971). The reservoir hosts and vectors of FISF are unknown, although on Flinders Island, *I. tasmanii* is the most common tick on humans and may be involved in transmission. The hard ticks, *Ixodes holocyclus*, *I. cornuatus* Roberts and *Ixodes tasmanii* Neumann are responsible for paralysis in humans and are confirmed vectors of *Rickettsia australis*, with native rats and bandicoots implicated as the host animals (Russell, 1998).

C. Ross River Virus in Tasmania and the Role of *P. gunnii*

6.9 INTRODUCTION

Australia has a diversity of vectors and vector-borne diseases with more than 70 arboviruses reported in Australia, but generally only a few have been of major concern (Mackenzie *et al.*, 1998). Ross River virus (RR) in particular is the cause of the most common human arboviral disease in Australia. In recent years concern has arisen due to the increase of mosquito-borne diseases possibly as a result of climatic changes across the continent. RR virus is a mosquito-borne alphavirus that produces a syndrome known as epidemic polyarthritis, the clinical disease often being referred to as Ross River virus disease (Mackenzie, *et al.*, 1998). The virus is endemic throughout much of northern and Central Queensland and epidemic in all states of Australia, Papua New Guinea and the Solomon Islands. The presence of RR virus in Tasmania was established in 1974, from a sentinel herd of Hereford cattle (McManus and Marshall, 1986). In 1977, a serological survey was performed on common native species, detecting the highest antibody levels in the Bennett's wallaby, pademelon, brush possum and common wombat (McManus and Marshall, 1986). In 1981, the first human infections were recorded from residents in the north of the State (Mudge *et al.*, 1981) and it has spread steadily southwards (McManus, personal communication). Since then, Tasmania and other temperate coastal zones report antibody detection in the human population to range between 6 – 15% of the community disease (Mackenzie *et al.*, 1998).

Recent surveillance programs have increased our understanding of the geographic regions, climate conditions and vector factors of arthropod-borne viral diseases, particularly for RR virus. Its activity is widespread but often localised with major outbreaks being reported throughout parts of Australia usually in association with heavy rainfall and/or high tides during summer months. (Amin *et al.*, 1998; Russell, 1998; Bielefeldt-Ohmann and Barclay, 1998). Surprisingly major metropolitan

areas of Australia have recently experienced increased viral activity normally considered to be associated with rural and semirural communities (Amin *et al.*, 1998).

Since 1990, several major epidemiological features of RR virus infection have been elucidated, recognizing that disease transmission is driven by mosquito abundance with various species involved. Laboratory studies have identified a number of species of *Aedes* as good vectors of RR virus (Russell, 1998), though findings by Lindsay *et al.*, (1993) and Dhileepan *et al.*, (1996) (both cited by Mackenzie *et al.*, 1998), included possible vertical transmission in desiccation–resistant *Aedes* eggs. Lindsay *et al.*, (1993) and Sammels *et al.*, (1995) (cited by Mackenzie *et al.*, 1998), described distinct topotypes of RR virus genetic variants within defined geographic regions. The two major proven mosquito vectors of RR virus, are *Culex annulirostris* and *Aedes vigilax* and these were thought to be absent from Tasmania, until McManus, (1994) first identified *Culex annulirostris* in this state. Whether this species is indigenous to Tasmania remains unknown. McManus, (1994) yielded two strains of RR virus from *Aedes flavifrons,* a fresh-water breeding mosquito. Equally abundant was *Aedes camptorhynchus* an efficient laboratory vector and considered a possible natural vector.

The major vertebrate hosts of RR virus are believed to be macropod marsupials (Aldred *et al.*, 1990; Vale *et al.*, 1991) though serological surveys have demonstrated the occurrence of infection in a wide range of native and domestic animals. Horses have often been considered to act as amplifier hosts in semi-urban areas (Gard *et al.*, 1977; Cloonan *et al.*, 1982). Grey Kangaroos and Agile Wallabies have been reported as developing a viraemia from infection with RR virus with no ill effects after infection (Kay *et al.*, 1986 cited by Spearet *et al.*, 1989). With the identification of two mosquito species, *Aedes flavifrons* and *Aedes camptorhynchus,* the potential for RR virus outbreaks in Tasmania is firmly

established (McManus, personal communication). As urbanisation begins to encroach on Tasmania's coastal and rural areas and with residential homes often bordering on reserves, a closer interaction between humans, native animals and arthropod vectors will undoubtedly occur. Bandicoots are often found nesting in introduced grasses and shrubs bordering on residential areas and farm properties. During this study it was noted that *P. gunnii* nests contained mosquitos and other ectoparasites which suggested that the Peramelidae are possible reservoirs of RR virus in Tasmania. With this observation it was decided to test *P. gunnii* sera for antibodies to RR virus.

6.10 MATERIALS AND METHODS

6.10.1 Serology

Fifty-eight serum samples collected from *P. gunnii* trapped at Huonville and the Grove region of southern Tasmania were tested for RR virus antibodies. Blood samples were collected from all animals by lancing the lateral ear vein. Approximately 0.25 ml of blood was collected into a Microtainer serum separator tube (Becton Dickinson & Co., Rutherford, New Jersey, USA) with a gel interface. Tubes were centrifuged and serum stored at –20°C until required. All samples were sent for RR virus serology using the Biocene Ross River Virus Total Antibody DEB-ELISA (Cat code: BIO-RRT. Biocene, NSW, Australia). The test was performed by Linda Hueston, at the Department of Microbiology, ICPMR building, The New Childrens Hospital, Westmead, NSW Australia.

DEB-ELISA is a Defined Epitope Blocking enzyme immunoassay that detects a single type specific neutralising epitope. The test measures total antibody and is species independent. Test methodology is outlined in Appendix 11.6.

	P. gunnii	RR virus		*P. gunnii*	RR virus
1	G 110	_	**31**	G 781 M	_
2	G 908	_	**32**	G 51 F	_
3	G 96	_	**33**	H 96	_
4	H 5	_	**34**	H 231	_
5	G 115 M	_	**35**	G 102	_
6	G 402 F	_	**36**	G 4	_
7	H 904 M	_	**37**	H 200	_
8	G 152 M	_	**38**	H 24 M	_
9	G 221 F	_	**39**	H 501 M	_
10	G 528 M	_	**40**	H 7	_
11	H 06	_	**41**	G 119 M	_
12	H 4	_	**42**	H 5 M	_
13	G 72	_	**43**	G 02	_
14	H 805 M	_	**44**	G 40	_
15	H 21	_	**45**	G 189 M	_
16	H 2	_	**46**	G 196	_
17	H 1	_	**47**	H 408	_
18	G 23	_	**48**	H 125	_
19	H 134	_	**49**	H 80 F	_
20	H 34	_	**50**	H 806 F	+
21	H 806	_	**51**	G 36 F	_
22	H 800	_	**52**	G 165 M	+
23	H 801	_	**53**	H 172	_
24	H 96 M	_	**54**	H 800	_
25	H 100	_	**55**	G 022	_
26	H 62 M	_	**56**	G 516 M	_
27	H 2	_	**57**	G 44	_
28	H 41	_	**58**	H 6	_
29	H 67	_	**59**		
30	G 50 M	_	**60**		

Table 6.3 *P. gunnii* tested for Ross River virus antibodies

KEY: Trapping site, **G** = Grove, **H** = Huonville,

F = **Female,** M = **Male**

6.11 RESULTS

Of the 58 samples tested, two (3.4%) *P. gunnii* were detected with RRV antibodies, animals G165 and H806 (Table 6.3). Titre levels were considered Low Positive at 1:8 and 1:16. As the test does not distinguish between IgG and IgM antibodies, but detects both, it cannot be determined if these results are a recent of past infection but they do indicate exposure and infection at some stage.

6.12 DISCUSSION

Due to the nesting behaviour of bandicoots, they have often been reported with mosquitos and ticks in their shelters. Vale, (1991) found a high prevalence of bandicoots in NSW with RR virus antibodies. McManus, (1994) first reported bandicoots in Tasmania with RR virus antibodies, a finding confirmed for the *P. gunnii* in the present study. The prevalence of serum antibodies suggests that the Peramelidae, including *P. gunnii*, are important potential reservoirs of RR virus given their widespread occurrence in coastal areas of eastern Australia, northern Queensland and Tasmania. The effects on *P. gunnii* of the RR virus are at present unknown, but the fact that they can become infected with this virus indicates that they, with other marsupials, can become a reservoir for the infection and be transmitted to humans via the mosquito vector. RR virus is the only presently known arbovirus infection of humans in Tasmania.

Chapter 7. Haematological and Biochemical Data of *P. gunnii*

7.1 INTRODUCTION

The blood of marsupials and monotremes first attracted the attention of Davy (1840) and Hobson (1842) (cited by Parsons *et al.*, 1971), who examined the various types of cells in these groups of animals. Interest then lapsed until Ponder *et al.*, (1928) and Briggs, (1936) examined further material. Haematological studies on marsupials and monotremes developed slowly until interest was revived by Agar *et al.*, (1976) who commenced the study of red cell enzymes and biochemistry of marsupials. This led to the more intense comparative study of metabolism and antioxidant defence systems in the erythrocytes of Australian marsupials and monotremes, as seen by the work of Parkinson *et al.*, (1995) and Whittington *et al.*, (1995). Within these reports very little if any study has included *Perameles gunnii.* Scientific publications providing normal values for blood parameters on bandicoots are sparse and are mainly derived for the Long-Nosed Bandicoot, *Perameles nasuta* and Short-Nosed Bandicoot, *Isoodon macrourus* (Agar *et al.*, 1976; Agar and Gemmell, 1993). A collection of data, including haematologic and serum biochemistry, is retained and updated for *P. gunnii* of Victoria at the Melbourne Zoological Gardens (Lynch, personal communication).

Baseline characteristics of haematologic and serum biochemical parameters can provide valuable source of data for zoos, management centres and veterinarians who are often consulted on clinical problems. For marsupials, especially vulnerable species such as *P. gunnii*, such analyses help recognise and monitor disease processes. Baseline data of this sort can provide an insight into the physiology and pathological alterations in wild and captive individuals, animal populations and allow a comparison with other species. Analysis of the peripheral blood film can

provide valuable information for the detection and description of medical conditions, where changes in haematological profiles often signal or indicate specific problems.

Canfield (1998) summarised the important interpretations of the peripheral blood slide for exotic and native animals, including detection of erythrocyte alterations and immature forms, proportions and appearance of leukocytes, platelet alterations and detection of blood parasites. It must be noted within these interpretations there is a degree of variability which must be considered and it should be appreciated that a variety of factors including stress can affect the results of such haematological studies (Whittington and Grant 1983; Smith, 1992). This current study provides the starting point of data collection for *P. gunnii* in Tasmania.

7.2 MATERIALS and METHODS

Haematological values and biochemical parameters for *P. gunnii* were derived from wild and captive animals. The collected data represents the physiological and pathological findings of *P. gunnii*, under conditions which were somewhat restrictive and limited to trapping seasons. To minimise the potential variation and confounding factors, consistency in the site and method of blood collection was important for all animals and essential in obtaining reliable haematological and biochemical values. Manual staining and cell counts were performed where ever possible. In general, analysis of blood and serum was performed using standard automated techniques.

7.2.1 Making and staining blood smears with Leishman's and Leishman's / Wright's mixtures.

Blood films were stained with a Romanowsky stain. Leishman's or Leishman's/Wright's stain was applied to the films. Automated systems were occasionally employed using an Ames Hema-Tek Slide Stainer (England) at the Department of Haematology of the Royal Hobart Hospital (RHH). When applying

the Leishman's / Wright's stain the preferred choice was the conventional manner as described by Mitruka and Rawnsley, (1977) rather than the dip technique.

7.2.2 Haematological Data Analysis using Automated Systems Chapter 2.2

Manual differential leukocyte count and total white cell counts were carried out using a haemocytometer, as described in Chapter 2; 2.2.

Blood samples were not analysed if considered unsuitable ie clotted or collected in field longer than recommended time. The following were recorded using the Technicon H2 Automated Counter (Dublin, Ireland), adapted to work with animal blood by adjusting mobile volume discriminators; white blood cells (WBC), red blood cells (RBC), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), haemoglobin distribution width (HDW) and platelet count (PLT) were recorded, mean platelet volume (MPV), platelet distribution width (PDW), and platelet crypt (PCT). HCT was measured, by centrifuging blood for 5 min in a microhaematocrit tube using a haematocrit centrifuge (Micro-Haematocrit Centrifuge, Hawksley, UK), in order to adjust the values obtained with the analyser.

Statistical analysis was performed with statistics software SPSS version 8 (SPSS Inc., Chicago, Ilinois, USA). Differences were compared among groups using a Mann-Whitney U test (Siegel, 1956). The minimum significance value chosen was $P \leq 0.05$.

7.2.3 Measurements of Cells Using the Image Analyser (Chapter 2; 2.2)

Measurements of cell size were performed using peripheral blood slides and utilising Image Analysing equipment as described in Chapter 2; 2.2

7.2.4 Scanning Electron Microscopy (Chapter 2; 2.3)

SEM was used to measure size and visualise any structural deformation in infected erythrocytes or changes to its membrane.

7.2.5 Haemoglobin Electrophoresis and HbF Quantitation

Haemoglobin F (Hb F), is the predominate haemoglobin formed during liver and bone marrow erythropoiesis in the foetus of mammals. Haemoglobin F composes 90 – 95% of its total haemoglobin. In adults, haemoglobin A (Hb A) consists of two chains and is the major haemoglobin in most adult species. Often the switch from Hb F to Hb A during development is incomplete causing haemoglobinopathies or anaemia, with increased levels of Hb F (McKenzie, 1988). Haemoglobin F can be frequently elevated in other haemotological disorders as well as in haemoglobinopathies. It is here an attempt is made to evaluate the status of *P. gunnii*. Electrophoresis allows the common structural haemoglobin variants to be seen on cellulose while alkali denaturation allows the amount of Hb F to be expresses as a percentage of the total haemoglobin.

Electrophoresis is considered the best method for separating and identifying the haemoglobins present in a blood sample (McKenzie, 1988). Haemoglobin carries an electrical charge resulting from the presence of carboxyl and protonated nitrogen groups and many amino acid substitutions alter this electrophoretic charge of the molecule, enabling detection of the structural haemoglobin variant by haemoglobin electrophoresis. Initial electrophoresis was performed using a red blood cell lysate in alkaline buffers and cellulose acetate and agar gel at pH 6.2 (Marder and Conley, 1959) as the major support medium used to yield the rapid separation of HbA, F, S and C and other mutants with minimal preparation time.

Blood samples of *P. gunnii* were collected in EDTA and processed following laboratory protocols of the Department of Pathology, Royal Hobart Hospital. A

haemolysate preparation was required before performing haemoglobin electrophoresis. To prepare a haemolysate a 3 mL blood sample from *P. gunnii* was centrifuged at 1900 x *g* for 5 minutes to remove plasma and buffy coat. The erythrocytes were washed three times in 0.9% saline following centrifugation. An equal amount of Tris buffer (0.005 M, pH 7.5) containing a trace of saponin was carefully added. Two drops of haemolysate reagent was added. The reagent contained 0.005 EDTA in purified water with 0.01% potassium cyanide added as a preservative (Cat. No. 5125, Helena Laboratories, Texas, USA). Standard haemolysate concentration must be between 9-11 g / dL.

The resulting haemolysate was centrifuged at 11,000 x *g* for 20 min, to remove cell debris and nuclei and adjusted to approximately 10 g / dL with Tris buffer. Electrophoresis was performed on cellulose acetate using 0.05M Tris-EDTA-borate buffer at pH8.6 (Cat. No. 5802, Helena Laboratories, Texas, USA). Good separation was obtained using 350V for 20 min at room temperature. The electrophoresis strips were stained with Pounceu S red (Cat. No. 5525, Helena Laboratories, Texas, USA) and cleared using a preparation containing 30 parts glacial acetic acid, 70 parts absolute methanol and 4 parts Clear Aid (Cat. No. 5005, Helena Laboratories, Texas, USA) a reagent containing polyethylene glycol. Comparison of *P. gunnii* and human control samples were compared.

For HbF quantitation, the percentage of haemoglobin fractions was obtained by alkali denaturation. For an evaluation of haemoglobin fractions the following methods were employed. The standard haemolysate was prepared at a concentration of 9-11 g / dL. A volume of 0.3 mL haemolysate was added to 5 mL of freshly made Drabkin's solution. It is important not to use commercial Drabkin's solution as it may contain alkali. Haemoglobin F is resistant to alkali and is not denatured by the addition of Sodium hydroxide. Hb A (and most other variants) are denatured by the alkali. Approximately 2.8 mL was transferred to a tube labelled "T" and 0.1 mL to a

tube labelled "C". The "T" tube was vortexed and 0.2 mL of Sodium hydroxide added into the centre of the liquid cone. This was vortexed for 15 sec. At exactly 2 min, 2 mL of saturated Ammonium sulphate was added into the centre of the vortex cone. The solution was filtered twice through the same filter paper. To tube "C" 6.8 mL of water was added. The OD of T and C tubes were measured at 420 nm against distilled water. If the OD of the T tube exceeded 1.0, water was added to dilute the sample. Calculations were performed for the percentage of Hb F present in *P. gunnii* by using the following equation.

$$\textbf{Equation}: \quad \frac{\text{OD T x Dil x 2.5}}{\text{OD C}} = \text{HbF}$$

7.2.6 Biochemistry

Biochemical analyses of blood were performed using standard automated techniques (Vitros System, 950; Johnson and Johnson, Clinical Diagnostics, New Jersey, USA) at 37°C. Biochemistry results were drawn from captive *P. gunnii* and wild caught animals. Captive animals were acclimatised to their food and environment for over a month. The animal's food and water intake were not strictly controlled. As these animals are nocturnal, animals were often bled late morning or afternoon, when feeding was not apparent. Wild caught *P. gunnii* specimens were collected during a capture and release investigation. These animals were likely to have been trapped during their feeding session. Traps were checked during early morning, therefore the effect of food would be considered minimal. The animals were selected by their appearance, body weight, shiny coat, and low ectoparasite load which together was deemed to indicate a good state of health. Minimum handling time was adhered to. It was difficult during field work to draw enough blood for large volumes of serum required to perform biochemistry. Approximately five, 1mL volumes of blood were collected in paediatric Microtainer serum separating tubes with a gel interface (Microtainer: Becton Dickinson & Co., Rutherford, New Jersey, USA).

Blood was left at room temperature and following centrifugation 2000g serum was transferred to glass tubes and analysed immediately or frozen (-10°C). Serum chemistry was evaluated on fresh samples. Occasionally samples were frozen and tested within 2 weeks of collection. The activities of enzymes were measured including alkaline phosphatase (Alk Phos) Gamma-glutamyl transpeptidase (GGT), alanine-amino transferase (ALT), aspartate aminotransferase (AST), Creatine kinase (CK), Lactate dehydrogenase (LD). The following assays were performed, by methods outlined in the Technicon 'manual' sodium, potassium, chloride, urea, creatinine, random glucose, total bilirubin, iron, and random cortisol, total protein, albumin, magnesium, serum osmolarity.

7.3 RESULTS

7.3.1 Peripheral blood films

Peripheral blood smears from *P. gunnii* provided a range of information, regarding erythrocytes, the types and relative numbers of white cells present, morphological features and characteristics of *P. gunnii* blood cell types, and bloodborne parasites. Amongst the leukocytes including neutrophils, lymphocytes, eosinophils, monocytes, the occasional unclassified cell and basophils were noted.

The red blood corpuscle of *P. gunnii* was a non-nucleated biconcave disc in conformity with its mammalian characteristics. Following the staining of peripheral blood smears with either Leishman's or Leishman's / Wright's it was common to find erythrocytes exhibiting what appeared to be polychromasia. The occasional late normoblast with Howell-Jolly bodies was noted, appearing as dense blue inclusions (Figure 7.1). This did not vary with haemoglobin content of the animal. Nucleated red blood cells were often seen in blood films (Figure 7.2). All cells were graded on a scale of 1-4+ as described by Canfield (1998). For *P. gunnii* the average grading of cells were approximately; 2+ polychromasia, 2+ Howell Jolly bodies and 4+ nucleated red blood cells.

Figure 7.1

Howell Jolly body and evidence of polychromasia (different colours) in a peripheral blood film of *P. gunnii*.

Leishman's Stain

Magnification: x 1200

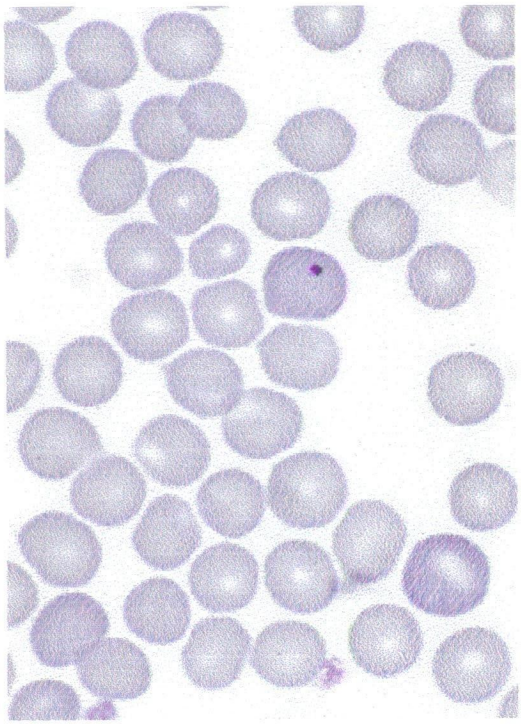
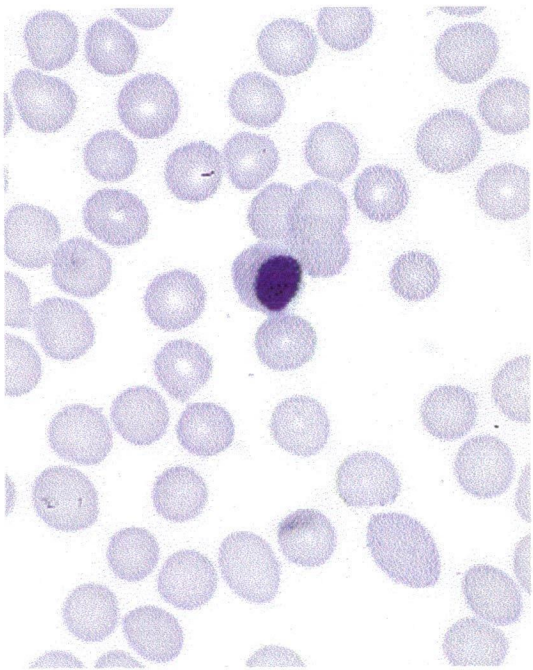


Figure 7.2

Normal *P. gunnii* blood film shows round erythrocytes, several with central pallor, and nucleated erythroid cell.

Leishman's Stain

Magnification: x 1000



Late normoblasts in which the nucleus was pyknotic were noted in some blood films giving the impression they were commencing to break up (Figure 7.3). Crenation of erythrocytes was evident in blood samples taken back to the laboratory following fieldwork (Figure 7.4). This feature is commonly reported for erythrocytes in prolonged contact with anticoagulant EDTA for many animal species (Canfield, 1998).

Lymphocytes were the predominant white blood cell in the peripheral blood of *P. gunnii*. They varied in size and in appearance, with larger and smaller cells detected and often within the same animal. The large lymphocytes appeared with more cytoplasm, and an indented oval nuclei. The cytoplasm contained azurophilic granules (Figure 7.5). Smaller lymphocytes were noted (Figure 7.6). It was difficult in some instances to distinguish between a lymphocyte and plasma cell. What appear to be mature plasma cells with binucleate features were often seen, being either classified as lymphocytes or monocytes (Figure 7.7).

Neutrophils were prominent in blood smears and often presented with variable morphological features. Circulating immature neutrophils or band neutrophils were commonly identified in blood slides by their plump nuclei, smooth contours and bluer cytoplasm (Figures 7.8 and 7.9). The nuclei are described as 'S' or 'U' shaped with no prominent indentation (Canfield and Martin, 1998). Metamyelocytes or earlier forms of neutrophils were often present, with the "ring" type of nucleus, a "doughnut" shape rather than the multilobulated nucleus (Figures 7.8 and 7.10). Hypersegmented neutrophils were often detected (Figures 7.11 and 7.12).

Eosinophils due to their known fragility were occasionally broken or distorted while making the smear (Figure 7.13), often presenting as fragments with released granules. In a normal smear, eosinophils contained nuclei with only two lobular masses and were surrounded by mature granules which stained red. Individual

Figure 7.3

This *P. gunnii* blood film shows the presence of a pyknotic cell.

Leishman's Stain

Magnification: x 1000

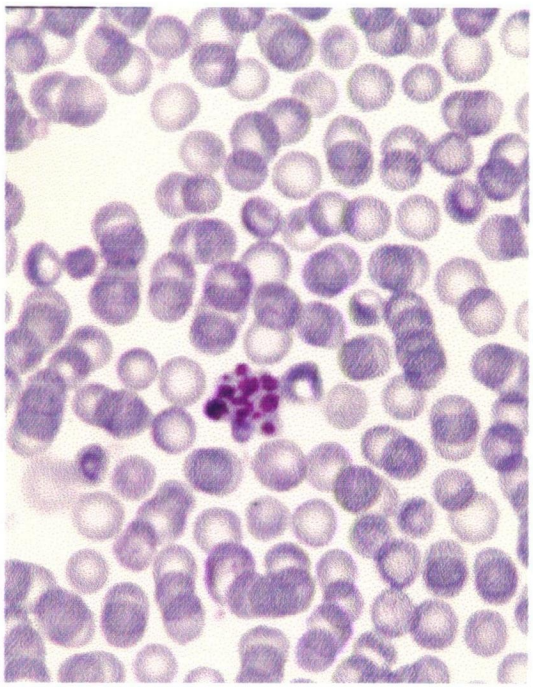


Figure 7.4

This *P. gunnii* blood film shows crenated erythrocytes surrounding a eosinophil. Crenated cells were induced by prolonged contact with EDTA.

Leishman's Stain
Magnification: x 1000

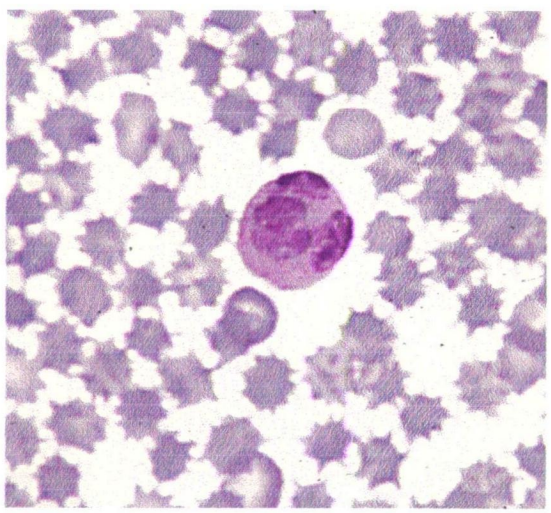
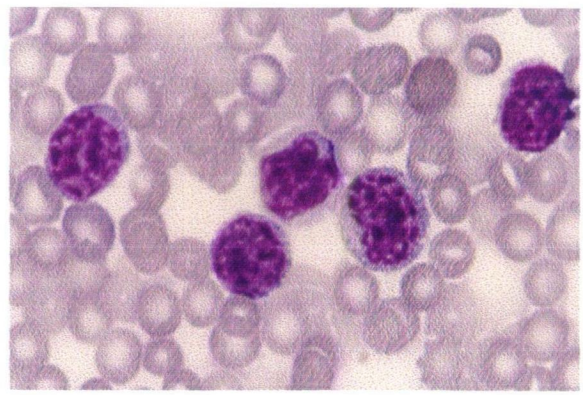


Figure 7.5

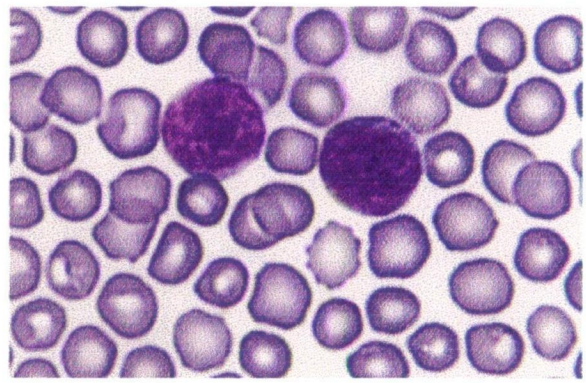


A cluster of large lymphocytes with azurophil cytoplasmic granules seen in a *P. gunnii* blood film.

Leishman's/Wright's Stain

Magnification: x 1000

Figure 7.6

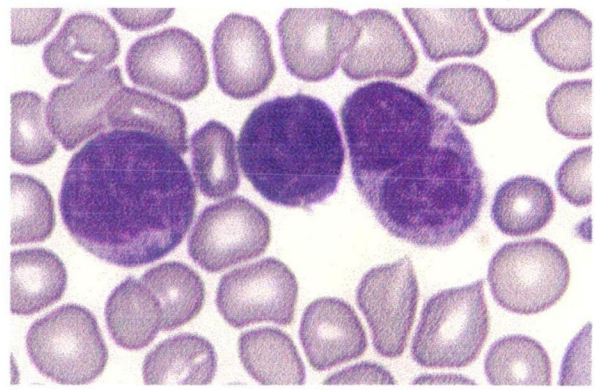


This *P. gunnii* blood film shows small lymphocytes with intense nuclei staining.

Leishman's/Wright's Stain

Magnification: x 1000

Figure 7.7

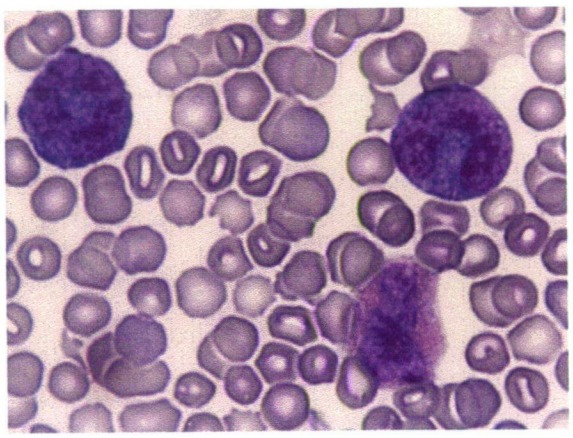


This *P. gunnii* blood film shows large lymphocyte, small lymphocyte and monocyte.

Leishman's/Wright's Stain

Magnification: x 1200

Figure 7.8

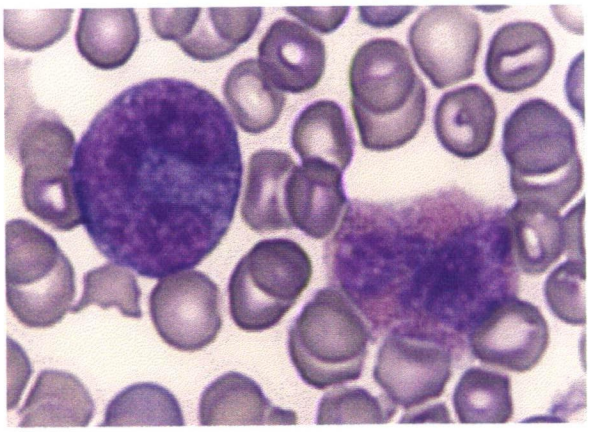


P. gunnii 'ring' metamyelocyte, 'band' neutrophil and distorted eosinophil commonly seen in *P. gunnii* blood films.

Leishman's/Wright's Stain

Magnification: x 1000

Figure 7.9

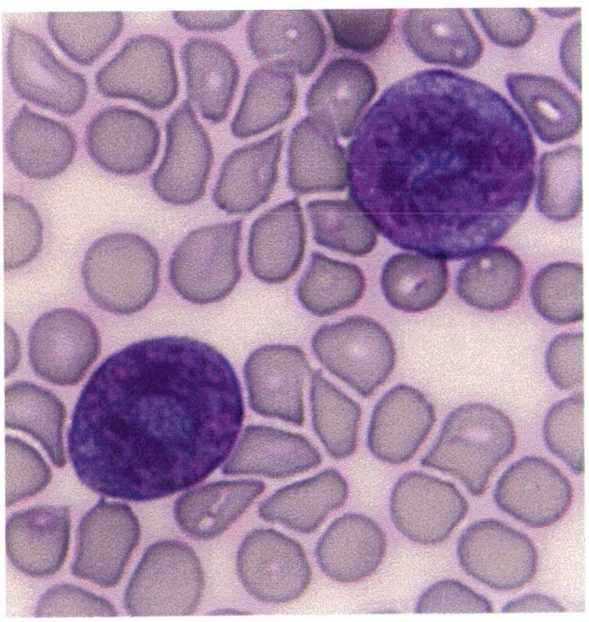


This *P. gunnii* blood film shows a segmented 'band' neutrophil and distorted eosinophil.

Leishman's/Wright's Stain

Magnification: x 1200

Figure 7.10

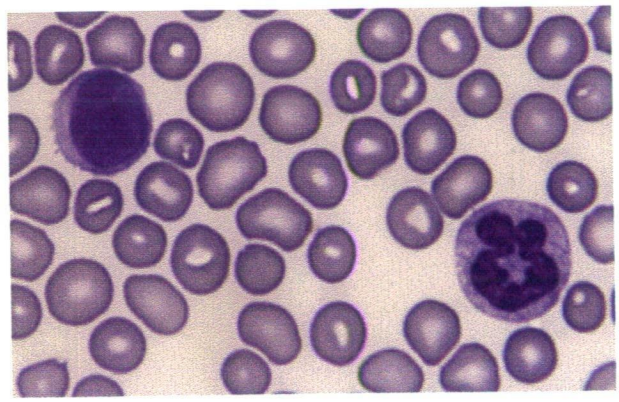


This *P. gunnii* blood film shows two 'ring' metamyelocyte.

Leishman's/Wright's Stain

Magnification: x 1200

Figure 7.11

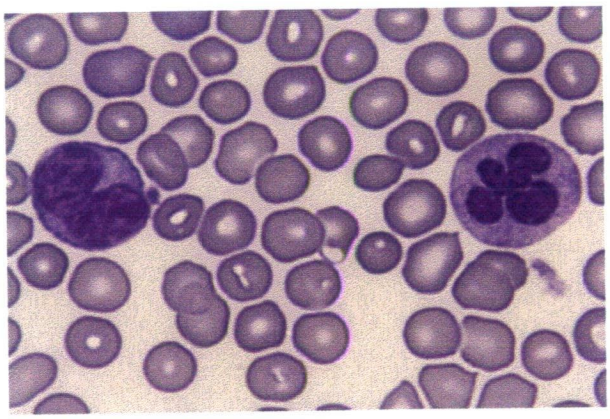


A lymphocyte and hypersegmented neutrophil seen in a normal *P. gunnii* blood smear.

Leishman's/Wright's Stain

Magnification: x 1000

Figure 7.12

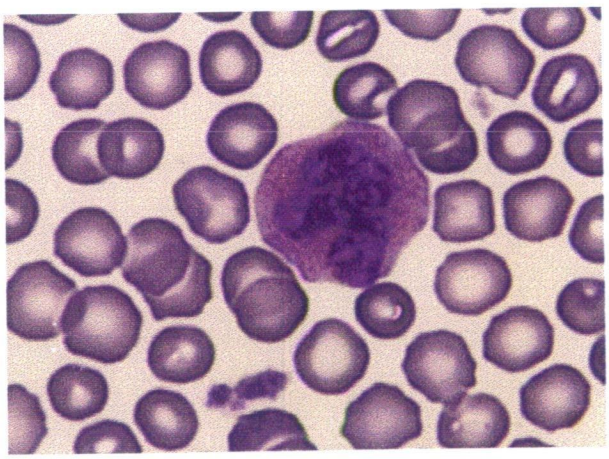


Monocyte and hypersegmented neutrophil seen in *P. gunnii* blood film.

Leishman's/Wright's Stain

Magnification: x 1000

Figure 7.13



Eosinophils were often seen to be distorted in *P. gunnii* blood films.

Leishman's/Wright's Stain

Magnification: x 1000

animals were detected with elevated eosinophil values, but there was no significant difference between animals with bloodborne parasites and those with no parasites in their peripheral blood.

Blood parasites *Hepatozoon* sp., and *Trypanosoma* sp., were detected in peripheral blood films. *Hepatozoon* sp., can occur as extracellular or intracellular forms. *Hepatozoon* sp., infected the erythrocytes of *P. gunnii* (Figure 7.14) and further description is found in Chapter 4.

7.3.2 Differential (percentage) Leukocyte Count

The average differential leukocyte count manually performed for non-parasite (bloodborne parasites) and parasite-infected animals is summarised in Table 7.1 and Table 7.2. The differential percentage of leukocytes from *P. gunnii* were; neutrophils 35.9%; lymphocytes 55.3%; monocytes 3.6%; eosinophils, 5.0%, basophils 1.0% Differential leukocyte values of animals with detected bloodborne parasites were; neutrophils, 35.7%; lymphocytes 54.8%; monocytes 3.3%; eosinophils 6.0%, basophils 1.0%. These parameters were analysed using a t-test on raw data. All other differences seen between these two groups could be reasonable attributed to chance.

7.3.3 Total white cell counts.

Erythrocyte values exhibited narrow ranges, but there was a degree of individual variability in counts of white blood cells. Total white cell counts ranged from 1.6 x 10^9 – 6 x10^9 / L with an overall mean and standard deviation of 4.4 x 10^9/L $\pm$ 1.21 x 10^9/L. There was some variation between manual cell counts and automated cell counts.

7.3.4 Automated Haematological Measurements.

Thirty blood samples collected were considered suitable for processing, using automated techniques. Samples were processed within 2 hours of collection but only

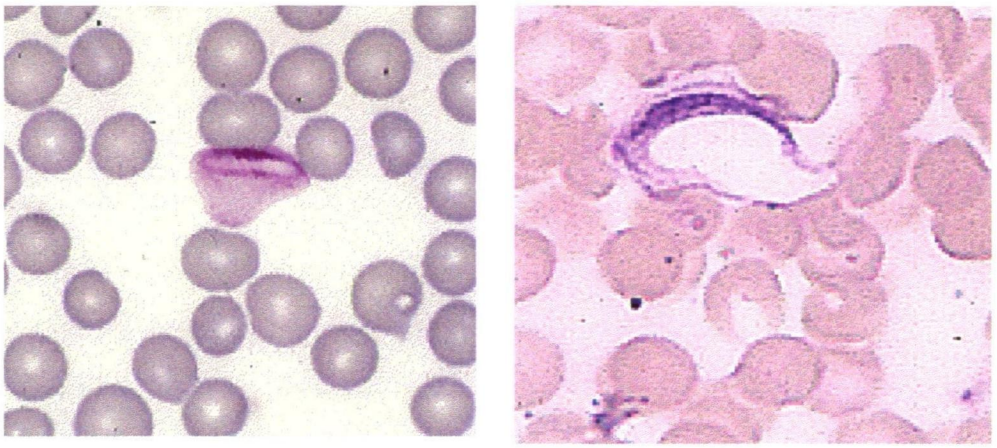


Figure 7.14

This *P. gunnii* blood film shows an intracellular *Hepatozoon* gametocyte in an erthrocyte and a trypanosome.

Leishman's Stain

Magnification: x 1000

	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophil
	35.9%	55.3%	3.6%	5.0%	0.6%
Mean	35.947	55.342	3.684	5.026	0.648
Std.Dev.	18.902	19.832	4.281	3.894	0.989
Std.Error	3.066	3.217	0.695	0.632	0.160
Count	38	38	38	38	38
Minimum	10.000	15.000	0.000	1.000	0.000
Maximum	78.000	82.000	20.000	19.000	3.000

Table 7.1 Differential (percentage) and values of leukocyte count of non-parasite infected *P. gunnii.*

	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophil
	35.7%	54.8%	3.3%	6.0%	0.4%
Mean	35.727	54.818	3.364	6.091	0.364
Std.Dev.	22.508	21.531	2.157	3.177	0.674
Std.Error	6.786	6.492	0.650	0.958	0.203
Count	11	11	11	11	11
Minimum	12.000	15.000	0.000	1.000	0.000
Maximum	77.000	74.000	7.000	11.000	2.000

Table 7.2 Differential (percentage) and values of leukocyte count of parasite infected *P. gunnii.*

nineteen samples were identified being free of bloodborne parasites. Baseline haematological values for *P. gunnii* sampled are described in Table 7.3. The data presented are from animals of normal health status, of adults and of both sexes. Comparison for sex-specific variations was analysed using Mann-Whitney U-test and Student's t-test assuming unequal variance and presented in Table 7.4. RBC, HGB, HCT, MCV, MCH, MCHC and PDW were all normally distributed. These parameters were analysed using a t-test. WBC, RDW and PCT were not distributed normally but there was only evidence of a statistical difference in RDW (red cell distribution width) between males and females ($p = 0.015$). HDW, PLT and MPV were all normally distributed when data was transformed to a log scale. These parameters were analysed using a t-test on the transformed data. All other differences seen between these two groups in all other blood parameters could be reasonable attributed to chance. Comparisons of this group with animals identified with bloodborne parasites are described in Chapter 4.

7.3.5 SEM and Measurements Using Image Analysis.

The red blood corpuscle was a non-nucleated biconcave disc in conformity with its mammalian characteristics (Figure 7.15). The diameters of various cell types were performed with Image Analysing equipment. The diameter of red blood cells from *P. gunnii* varied between 5.0 – 6.5 μM, with an average diameter of 5.6 μM. Neutrophil diameter ranged from 10.8 - 17.7 μM with an average diameter of 11 μM. Lymphocytes were between 8.0 – 12.2 μM with an average diameter of 9.5 μM. Eosinophil diameter was recorded between 10.8 – 17.6 μM with an average of 13 μM. Basophil diameter ranged between 11.34 – 21.54 μM with an average of 14.3 μM.

7.3.6 Haemoglobin electrophoresis and Hb F detected

Thirteen *P. gunnii* samples were processed for haemoglobin electrophoresis. Samples were run with two human controls. Cellulose acetate electrophoresis

	No.	Mean	Median	Maximum	Minimum	25th Percentile	75th Percentile
WBC x10^9/L	9	5.07	4.04	9.78	1.90	2.33	8.68
RBC x10^{12}/L	19	7.76	7.67	9.62	6.21	7.01	8.38
HGB (g/L)	19	141.89	143.00	177.00	115.00	133.00	149.00
HCT (L/L)	19	0.40	0.41	0.50	0.28	0.35	0.44
MCV (fL)	19	52.29	52.10	55.00	49.40	51.30	53.40
MCH (pg)	19	18.42	18.20	21.80	16.30	17.90	18.50
MCHC (g/L)	19	349.00	345.00	394.00	325.00	336.00	356.00
RDW (%)	19	14.33	14.20	16.60	13.30	13.60	14.90
HDW (g/L)	19	23.91	23.50	27.30	21.10	22.60	25.30
PLT x10^9/L	19	116.42	105.00	326.00	22.00	53.00	155.00
MPV (fL)	19	4.57	4.20	8.50	2.70	3.20	5.70
PDW (%)	18	80.23	82.05	100.60	56.20	71.20	90.20
PCT (L/L)	17	0.014	0.001	0.070	0.000	0.001	0.020

Table 7.3 Descriptive Statistics of baseline *P. gunnii* haematological values.

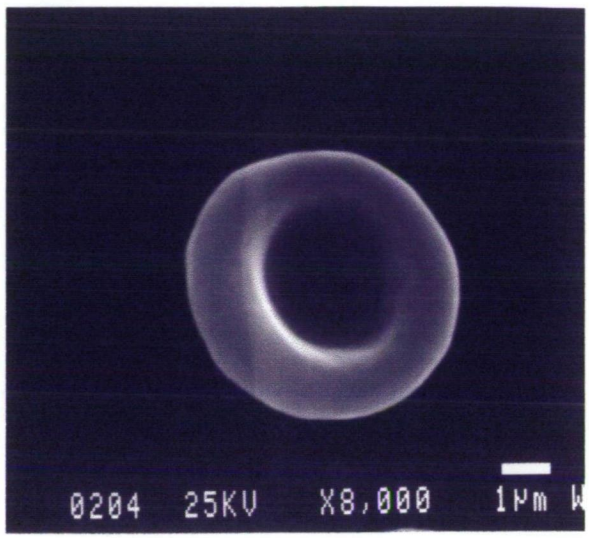
Data was analysed using Mann-Whitney U-test	
	p-value
WBC	0.121
RDW	0.015
PCT	0.726
Data analysed using a t-test assuming unequal variance	
	p-value
RBC	0.096
HGB	0.137
HCT	0.236
MCV	0.529
MCH	0.929
MCHC	0.229
LOGHDW	0.170
LOGPLT	0.927
LOGMPV	0.035
PDW	0.387

Table 7.4 Comparison of *P. gunnii* male and female haematological values.

Figure 7.15

Scanning electron micrograph of a red blood cell from *P. gunnii*

Magnification: x 8000



Test *P. gunnii*	Control	% HB F
0.058	0.061	2.37
0.042	0.083	1.26
0.047	0.102	1.15
0.078	0.15	1.3
0.058	0.103	1.4
0.069	0.075	2.3
0.043	0.111	0.11
0.053	0.073	1.82
0.099	0.049	5.05
0.032	0.087	0.92
0.056	0.1	1.4
0.075	0.086	2.18
0.063	0.062	2.54

Table 7.5. Percentage of Hb F present in *P. gunnii* using alkali denaturation.

BIOCHEMISTRY	Mean	Std Dev	Min	Max
Sodium (mmol/L)	142.86	3.43	139	148
Potassium (mmol/L)	6.46	0.48	6.0	7.1
Chloride (mmol/L)	113.71	3.08	110	117
Magnesium (mmol/L)	0.85	0.02	0.82	0.88
Urea (mmol/L)	11.94	0.94	10.5	12.9
Random glucose (mmol/L)	13.83	0.59	13.2	14.8
Creatinine (µmol/L)	43.71	5.98	33	59
Total bilirubin (µmol/L)	24.86	22.20	24	52
Total Protein (g/L)	66.43	3.92	62	71
Albumin (g/L)	34.71	5.59	29	40
AST (U/L)	83.43	3.13	79	88
CK (U/L)	267.86	117.38	161	494
LD (U/L)	5338.00	5555.45	8257	12639
Alk Phos (U/L)	283.57	30.80	236	293
GGT (U/L)	23.00	26.86	20	60
ALT (U/L)	86.86	66.54	25	173
Serum Osmolality (mmol/kg)	301.43	10.29	280	318
Iron studies (µmol/L)	57.86	4.45	50	62
Random Cortisol (nmol/L)	259.71	67.16	188	336

Table 7.6 Comparative biochemistry values of *P. gunnii*

KEY: Alk Phos - Alkaline phosphatase
GGT - Gamma-glutamyl transpeptidase
ALT - Alanine-amino transferase
AST - Aspartate-amino transferase
LDH - Lactate dehydrogenase
CK - Creatine kinase

revealed a single band a minor fast component with mobility approximating that of Human Haemoglobin S. A similar pattern was obtained by agar gel electrophoresis. The alkali resistant component could not be separated from the main component by changing pH or molarity of the buffers, or by increasing separation time. Hb F quantitation of *P. gunnii* detected ranges as low as 0.11% and as high as 5.05% (Table 7.5). The normal range for humans is less than 1.50% total haemoglobin.

7.3.7 Biochemistry

Biochemistry of *P. gunnii* sera including plasma enzymes analysed are presented in Table 7.6. Values from captive and wild caught animals were not consistent in any particular way, minimum and maximum values highlight individual variations. Results were comparable to [illegible] values of *P. gunnii* held captive in Victoria (Appendix 11.7). LDH values were particularly variable. Total bilirubin appeared elevated in all animals tested.

7.4 DISCUSSION

With little to no quantitative information available about the haematology of free-ranging *P. gunnii* of Tasmania, results were generally similar to collected data of captive *P. gunnii* from Victoria. The haematological data represent a baseline for free-ranging *P. gunnii* that may be used as a reference for long-term monitoring of the health of the Tasmanian *P. gunnii* population and as a tool for rehabilitation facilities. A compendium of normal values on marsupials will be maintained for reference data with the prospect of additional data.

Cell morphology varied remarkably across the peripheral blood smears examined. Blood films of *P. gunnii* often exhibited polychromasia which indicated that the cell was taking up both the basic and acid dyes due to the alteration in the haemoglobin content of the erythrocyte. This has been seen in other animal species, and in human reports of polychromasia, the cell exhibits much the same colouration as seen in the

intermediate stage of erythropoiesis (Mitruka and Rawnsley, 1977). The cytoplasm lags behind the nucleus in maturation and has not completely lost its ribonucleic acid. Dogs with regenerative anaemia commonly present with polychromasia, reticulocytes, spherocytes or nucleated erythroid cells (Canfield and Martin, 1998). Clinical assessment of *P. gunnii* was limited but haemoglobin levels in the animals were on average comparable results to *P. gunnii* of Victoria.

Numerous Howell-Jolly bodies were observed in blood smears of *P. gunnii*. In cats and dogs, Howell-Jolly bodies indicate splenic disease, as the spleen is responsible for 'pitting' remnant nuclear material (Canfield and Martin, 1998). It appears nuclear maturation lags behind cytoplasmic haemoglobulination, so the nucleus condenses usually in the late stages. In humans, Howell-Jolly bodies are nuclear remnants usually being seen in patients with megaloblastic anaemia or leukaemias (Mitruka and Rawnsley, 1977). But these bodies are commonly reported in other marsupial species such as the Tasmanian Devil (*Sacrophilus harrisii*) and the Eastern Quoll, *Dasyurus viverrinus* (Melrose, personal communication). As the spleen is responsible for their removal it may suggest *P. gunnii* have this limited function, though the interrelationship between the Howell-Jolly bodies and nucleated red cells is not immediately obvious.

It was illustrated that there were occasional difficulties in identifying polymorphonuclear cells with any degree of certainty. The lack of distinctive granules often suggested the cells were either monocyte or large lymphocytes. On the other hand they could have been cells related to the neutrophil. This feature is frequently difficult in other animals and occasionally in humans (Reith and Ross, 1977).

The distribution of nuclear lobulation in *P. gunnii* neutrophils appeared a common characteristic of this species. In many marsupials the formation of lobes are

uniformly "doughnut-shaped" (Melrose, personal communication). The 'band' and 'ring' neutrophil were commonly seen.

Large and small lymphocytes were often seen, the former approximately the size of a normal neutrophil and sometimes containing dark staining azurophilic granules. The monocyte nucleus was variable in shape. Eosinophils were generally larger than the neutrophils, and the small, round strongly acidophilic granules filled the cytoplasm. Basophils were not common, or were absent in many individual *P. gunnii.*

Platelet numbers are affected by age and sex in many animal species (Smith *et al.*, 1994). The size of *P. gunnii* platelets were relatively small compared with the diameter of erythrocytes but occasionally large platelets were noted. Platelet aggregation appeared common when samples were improperly handled i.e., in the wild, and has been noted amongst exotic and native animals (Canfield, 1998). Typically, crenation of erythrocytes was present in a number of *P. gunnii* blood samples and likely to have resulted from prolonged exposure to anticoagulant EDTA. Canfield and Martin, (1998) describes such artefacts as common in the blood dogs and cats because of ageing e.g., spherocytic changes can occur in erythrocytes within 20-30 min of collection because of depletion of intracellular adenosine triphosphate.

During the investigation two new blood-borne parasites were identified in *P. gunnii* including the *Hepatozoon* sp., and *Trypanosome* sp. Both were the first record of such parasites reported from this species in Tasmania. Though there were no statistical differences between animals infected with bloodborne parasites and those uninfected, elevated numbers of eosinophils in individual *P. gunnii*, were attributed to parasitism and physiological stress, as parasitic infestation often cause increased platelet, lymphocyte and eosinophil counts (Mitruka and Rawnsley, 1977).

The haematological values for *P. gunnii* studied during this investigation compares closely to South American marsupial species and other mammalian species including

humans, using values from Mitruka and Rawnsley, (1977). A greater difference in individual haematological values are possibly attributable to physiological stress and parasitic infections and infestations. Differences have been observed between sexes of many species (Mitruka and Rawnsley, 1977); this difference was attributed to the higher red cell distribution (RDW) detected in male *P. gunnii* than in female *P. gunnii.* These differences may be of physiological origin, such as excitement and movement during restraint.

Turk's solution was suitable for most *P. gunnii* analysed in performing manual differential leukocyte counts. The variation between total white cell counts and white cell counts using automated system has often been attributed to nucleated red cells (Melrose, personal communication) that may interfere with detection and analysis.

It was important to calibrate the automated cell counter to obtain accurate red cell counts and mean cell volumes especially for animals with small red cells. In many cases data from automated analysis of *P. gunnii* was not used. Any distinctly high values threw suspicion on the result. Manually-derived counts, including a centrifuged microhaematocrit was more consistent with the blood picture. Inherent shortcomings of particular analytical systems has been reported by other workers, as interference by one cell type being counted in an inappropriate channel can occur in a variety of circumstances (Smith *et al.*, 1994). When testing for *P. gunnii* numerically valid differentials were often reported even when the software failed to fully gate all the leukocyte populations. If warning 'flags' were generated samples were manually screened. It was essential to examine blood films to confirm abnormal data.

Measurements of *P. gunnii* red blood cells were performed using image analysis and scanning electron microscopy. Red cells measured an average 5.6 μM in diameter using image analysis and approximately 5.8 μM using scanning electron

micrographs. Comparative values between image analysis and scanning electron micrographs were not performed. Representative examples of scanning electron micrographs of marsupial red blood cell compared with human red blood cell have been presented by Benga *et al.*, (1992). Benga *et al.*, (1992) studied the red blood cell of the bandicoot, *Isoodon macrourus* and other marsupial species, describing all these animals red blood cells as biconcave discs, the only morphological variation being the size of the cells. Red blood cells of the Tasmanian devil, (*Sarcophilus harrisii*), bilby, bandicoot and Goodfellow's Tree kangaroo (*Dendrolagus goodfellowi*) were around 7 μM in diameter. Those of the koala were in the range of 7.8 - 8.6 μM. The animals described were all from diverse habitats with diverse food and body sizes.

The presence of an alkali resistant haemoglobin in the adult marsupial was first recorded by Parsons *et al.*, (1970) in the Tasmanian devil. Parsons *et al.*, (1971) revealed that this was a widespread feature of both the marsupials and the monotremes. But the levels recorded for the *P. gunnii* ranges of Hb F were as low as 0.11% and as high as 5.05%, which were lower than those obtained by Parsons *et al.*, (1970) who used the method of Singer (Singer *et al.*, 1951). The significance of the component and what is expected to be "normal", is difficult to define and further work needs to be done. The method of alkali denaturation as performed, is considered more accurate than by electrophoretic strip (Beck, 1992). No animals were reported carrying young but it must be considered that some of the female *P. gunnii* in this study could have been in the early stages of pregnancy which was undetected. Studies of pouched young would have to be undertaken to determine whether this component represents a residual amount of true "foetal" haemoglobin.

Cellulose acetate electrophoresis revealed one distinct band, a minor fast component with mobility similar to human Hb S. A similar pattern was obtained by agar gel

elecrophoresis. This was a similar profile recorded by Parsons *et al.*, (1971) for *I. obesulus*, the Southern Brown Bandicoot.

A biochemical profile was attempted in this investigation. Raised plasma levels of specific enzymes can be very useful indications of specific disease. But in many instances raised levels occur for reasons other than any pathology. Individual laboratory tests are subject to a number of variables which must be considered when making an interpretation or comparison. Physiological parameters between individuals fluctuate considerably, making the differences more difficult to explain. Diurnal, annual, dietary and postural variations in plasma constituents have been well document amongst humans and experimental animals (Smith *et al.*, 1994). This too can be applied to *P. gunnii*.

No bloodborne parasites and no extreme haematological values were detected in the animals selected for biochemistry. Of the bandicoots analysed the average total bilirubin levels of 37 μmol / L were slightly higher than the average value of *P. gunnii* from Victoria. An overproduction of bilirubin can be caused by several factors, the most common being an increased rate of destruction of circulating red cells or haemolytic anaemia. There could be an abnormal hepatic haeme metabolism or ineffective erythropoiesis originating in the bone marrow from defective erythrocytes and their precursors. In wild possums, bilirubin levels are lower in summer and autumn than winter and spring (Viggers and Lindenmayer, 1996). No seasonal changes were recorded in this investigation but these values may be normal for *P. gunnii* as the animals appeared to be healthy and there was no concurrent increase in other hepatic enzymes, which would be expected if there was any associated liver pathology. This can be discussed for LDH, seasonal, diurnal and strenuous activity having been reported in variations of LDH activity in a range of animals (Henry *et al.*, 1974). Similarly increased muscle enzymes such as ALT,

AST, CK have often been attributed to stress of physical capture in animal species such as wildcats (Fuller *et al.*, 1985; Marco *et al.*, 2000).

The total protein levels of Tasmanian *P. gunnii* were elevated in comparison to the Victorian species. In many species, what constitutes normal protein content of serum appears to be difficult to define, and therefore difficult to interpret for *P. gunnii.* The average total protein for the *P. gunnii* sampled was 66 g/L, which falls between the human averages. What might affect total serum protein concentrations in species profiles have included many factors, such as serum *versus* plasma samples as opposed to venous *versus* capillary serum which appears to make no difference (Mitruka and Rawnsley, 1977). The choice of anticoagulants may make a minor difference. The health of the animal is an obvious determinant, as total serum protein levels can alter if samples are taken with the subject supine or standing up. Exercise, age, meals and sex show little if any difference. Pregnancy and some drugs show alterations in total serum protein (Mitruka and Rawnsley, 1977).

An interpretation of "normal" or "abnormal" is based on the limits of "normal" established in the laboratory. With wild caught animals undiagnosed pathological conditions or borderline cases and chronic infection in the selected healthy population may have significant effects on the test result. Physiological and biochemical studies from America, suggest that in some instances the variety of internal parasites in clinically normal opossums causes difficulty in establishing correct normal values in this species (Timmons and Marques, 1969). Seasonal differences have been detected for several biochemical parameters of marsupials especially amongst possums (Viggers and Lindenmayer, 1996). Elevated dietary variations in serum urea levels have been reported amongst marsupial species.

Analytical variations were often difficult to minimize. It was not always possible to use replicate standard sera or analysis of the same specimen with different test

methods at different time intervals. Adults of different ages and sexes were used to determine the normal range of clinical biochemical and haematological values.

Standard methods of sample collection, storage and preparation were, whenever possible, strictly adhered to. Most values presented here are indicative of a mean value for the population available for testing. Although intraspecies physiological variation contributed to the range of biochemical and haematological values, it could be possible to minimize the variations by selecting populations of animals that are kept under constant environmental and nutritional conditions. Mitruka and Rawnsley, (1977) comment that in some instances when dealing with laboratory animals, selecting genetically pure populations of animals is preferable.

With this in mind, the current work brings forward several aspects that can be considered while assessing and evaluating the health status of individual and populations of *P. gunnii.* As more animals are tested and data collected, a better picture of *P. gunnii* and its health can be made.

Chapter 8. Profile of *P. gunnii* Immune System

8.1 INTRODUCTION

Reports of marsupial vulnerability to toxo-infection are numerous and range from clinical outbreaks and high mortality rates in zoos around the world (Dobos-Kovacs *et al.*, 1974, Boorman *et al.*, 1977; Jensen *et al.*, 1985; Dubey *et al.*, 1988b; Canfield *et al.*, 1990a) to overt disease in wild marsupial populations (Obendorf and Munday, 1983, 1990; Johnson *et al.*, 1988; Lenghaus *et al.*, 1990). Included in this susceptible group are the New World primates where infection with *T. gondii* often proves fatal (Cunningham *et al.*, 1992; Innes, 1997; Dietz *et al.*, 1997; Juan-Salles *et al.*, 1998). Amongst domestic and free-ranging animals, as well as humans, susceptibility to clinical toxoplasmosis is often considered to be a result of lowered immunity (immunosuppressive factors), stress-related conditions (environmental, physiological or population-based stress), debility, age or concurrent disease (Lenghaus *et al.*, 1990). At present, the reasons for the high susceptibility amongst the marsupials and New World primates are often attributed to these factors. Another common trait that Australian marsupials share is that they evolved largely separated from the cat, which is the definitive host of *T. gondii*. Consequently they have only recently become exposed to *T. gondii* and, as many marsupials succumb and die from toxoplasmosis, it is probable these animals have not evolved an effective immune response against this parasite.

Infection with *T. gondii* is widespread amongst warm-blooded animals and in the immunocompetent animal the infection generally produces no ill effects. The parasite and the immune system reach a compromise situation where the parasite encysts and remains in the tissues for the lifetime of the host, and the animal is protected against subsequent challenge. Nonetheless, the protozoan is an important pathogen in immunologically vulnerable hosts (Wong and Remington, 1993). When an animal is immunocompromised, such as humans undergoing chemotherapy for

cancer treatment or in HIV infected patients, *T. gondii* can act as an opportunistic infection and cause serious and sometimes fatal disease by infection, re-infection or reactivation (Seitz and Trammer 1998). As a primary infection, toxoplasmosis is a major concern to many pregnant animals resulting in abortion or severe congenital defects in the foetus (Esteban-Redondo and Innes, 1997).

Toxoplasmosis has been reported in wild *P. gunnii* which often fail to show any clinical signs or significant antibody levels before their sudden death (Pope *et al.*, 1957; Obendorf *et al.*, 1990), suggesting that these animals have impaired immunity. Host defence against *T. gondii* infection depends primarily on the cell-mediated arm of the immune system although humoral immunity is also critically involved. Experiments of adoptive transfer between chronically infected and naïve mice have shown that protection can be transferred by immune T-cells but not by serum (Khan and Kasper, 1996).

The innate immune response is responsible for the early detection and rapid destruction of *T. gondii* tachyzoites. Macrophages play an important role in phagocytosing the organism and, with the help of T-cells through the production of cytokines such as IFN-γ, are activated to become more efficient at phagocytosis (Suzuki and Remington, 1990; Beaman *et al.*, 1992). IFN-γ is therefore considered a critical component of protective immunity against *T. gondii* (Wong and Remington, 1993). During acute *T. gondii* infection, phagocytosis, which is normally protective, can lead to parasitaemia by dissemination of *T. gondii* engulfed within mononuclear phagocytes. Such a mode of spread appears to be advantageous to the parasite. As intracellular organisms, they are protected from the cytotoxic effects of antibody and complement. It appears that human and murine macrophages readily support their multiplication (Hauser and Tsai, 1986). As immune competent individuals do not succumb to overwhelming toxoplasmosis during the early stages of infection, other immune responses may be protective against the parasite prior to the reactivation of

the acquired immune response. There is evidence to support natural killer cells (NK) in this role (Dannemann *et al.*, 1989). The role of natural killer cells as non-specific effector cell types with cytotoxic activity against *T. gondii* extracellular tachyzoites has been demonstrated with murine splenic NK cells but not confirmed with human NK cells (Hauser and Tsai, 1986; Goyal *et al.*, 1988; Dannemann *et al.*, 1989). It has been suggested that resistance to toxoplasmosis may be associated with lymphokine-activated killer (LAK) cell activity (Wong and Remington, 1993).

The evasive strategies of *T. gondii* are complex but in immunologically competent hosts, immune response is partially effective in removing the tachyzoites during the acute stage of an infection, prior to the development of cysts. The tachyzoites are the main target for the protective immune response but as this is not always completely effective, cysts can develop and remain dormant and undetected (Channon and Kasper, 1996), with no evidence of inflammation (Sibley, 1993).

The complexities of the immune system have slowly been unravelled due to the intensity of basic research in human and laboratory animals. A trend toward more basic research in veterinary immunology, especially in domestic animals has been observed (Kristensen *et al.*, 1982), since Nowell (1960) described phytohaemaglutinin (PHA) as a stimulator of mitosis in human leukocyte culture and Pearmain *et al*, (1963) described the processes of lymphocyte blast transformation *in vitro*. Lymphocyte stimulation and proliferation studies have become an important biological tool for research in immunology. *In vitro* proliferation studies have contributed to the understanding of immune function in many species of animals including some marsupials (Kristensen *et al.*, 1982, Wilkinson *et al.*, 1992). The correlation and generalisation of the results obtained by researchers of vulnerable Australian animals such as the koala (Wilkinson *et al.*, 1992) and the analysis of those results may be used to make predictions regarding their immune status. But for many other marsupials, immunological data remains scant and incomplete.

The apparent disease susceptibility of *P. gunnii* and the fact that few immunological studies to date have been published from the viewpoint of comparative immunology, suggested that an analysis of aspects of both the cell mediated and humoral immune systems was necessary. Given the limited access to blood samples due to the small blood volumes that can be extracted from each animal and the number of animals available due to their endangered existence, this study concentrated on evaluating different components of the immune response that were most likely to be important for protection against infections such as toxoplasmosis. Innate immunity was evaluated by studying phagocytosis while the acquired immunity was evaluated by studying mitogen induced blast transformation for cell mediated immunity and *in vitro* antibody production for humoral immunity.

8.2 MATERIALS AND METHODS

8.2.1 Blood samples

All *P. gunnii* used in the study were young adults (700 - 790 g), live trapped from wild populations in Kingston (Tasmania, Australia: 43°S, 147 03'E) and Huon Valley (Tasmania, Australia: 43 03'S, 147 02'E) in accordance with established guidelines and protocols of Parks and Wildlife Service permit to take protected wildlife (Hobart, Tasmania, Australia; Permit No., 96253, 95249, 98166, 95256, 99054). Approval was received from the University of Tasmania Ethics Committee (Animal Experimentation; Investigation No., 94010, 95010, 95082, 98016).

8.2.2 A Rapid Whole-Blood Microassay for Neutrophil Phagocytosis. Labelling of bacteria with Fluorescein 5 Isothiocyanate (FITC)

Escherichia coli organisms were cultured for 18 hours at 37°C in nutrient broth (CM1, Oxoid Australia Pty Ltd, Victoria, Australia). The broth culture was centrifuged at 10,000 x *g* for 10 minutes. The supernatant was discarded and the pellet of bacteria washed with Dulbecco's phosphate-buffered saline (DPBS) (Gibco BRL, Life Technologies, Victoria, Australia) and resuspended. Enumeration of the

bacteria was achieved by serial dilutions and plating onto nutrient agar plates (CM3, Oxoid Australia Pty Ltd, Victoria, Australia). Colony counts were correlated to absorbance values using spectrophotometry so that the final concentration was approximately 10^9 cells / mL. A bacterial suspension was made in DPBS to achieve the absorbance values recorded. The bacterial suspension was heat inactivated at 60°C for one hour. The bacterial suspension was washed and centrifuged twice at 1000 x *g* for 10 minutes at 22°C using a benchtop Sorvall centrifuge before being resuspended in DPBS to a final concentration of 10^9 cells / mL using spectrophotometry. Bacteria were then aliquoted into 1 mL centrifuge tubes and frozen at –70°C.

When required the bacterial stock was thawed and incubated with 25 µL of Fluorescein 5 Isothiocyanate (FITC, Sigma Chemical Company, St Louis, USA) at 1mg / mL on a rotator. After 2 hours the sample was washed in PBS and centrifuged three times at 1000 x *g*. The sample was centrifuged for approximately 1 minute to ensure no bacteria were lost. At this stage fluorescence was checked under a UV microscope (Leitz, Dialux 22EB, Germany). FITC labelled bacteria were opsonized with 100 µL of animal serum (host to be tested) for 15 minutes at 37°C on a rotator. Bacteria were then washed twice in DPBS (1 mL volumes) and resuspended in 100 µL. Suspensions were sonicated twice for 5 second intervals at low frequency.

8.2.3 A Rapid Whole-Blood Microassay for Neutrophil Phagocytosis.

Blood was collected from captive seronegative bandicoots into EDTA paediatric tubes (Becton Dickinson & Co., Rutherford, New Jersey, USA) and stored for no longer than 3 hours prior to use. More prolonged storage can result in suboptimal neutrophil (polymorphonuclear leukocyte)(PMN) isolation or altered phagocytosis. 100 µL of whole blood was washed in PBS and centrifuged twice at 800 x *g* to remove EDTA and plasma. Red blood cells were lysed using red blood cell (RBC) lysis buffer and white blood cell (WBC) counts were performed on an improved

Neubauer haemocytometer. If the WBC count exceeded 10^7 cells/ mL the whole blood was diluted in phosphate buffered saline to an estimated 10^6 cells / mL range.

1 mL of FITC labelled bacteria was sonicated to disrupt clumps and added to the washed whole blood samples. The mixture was incubated at 37°C on a shaker and incubated at appropriate time intervals. Phagocytosis was terminated by placing the samples on ice and 500 µL of icecold PBS was added to each sample. Samples were washed twice with this volume of PBS and microcentrifuged. For each blood sample, one tube with cells and no bacteria, was carried through as a control as well as one control sample of bacteria and FITC. 500 µL of RBC lysis buffer was added and sample placed on ice for 5 minutes or until lysis was visible. The sample was centrifuged and supernatant discarded leaving a white pellet that was resuspended by flicking the tube. 500 µL of PBS was added and centrifuged twice. Test samples were quenched with 200 µL PBS and 50 µL Trypan blue (0.25%). Following quenching cells were aliquoted into polystyrene round bottom tubes (Falcon 12 x 75 mm, Becton Dickinson and Company, New Jersey, USA) and run through the flow cytometer. Cells were also observed using fluorescent microscopy and light microscopy.

8.2.4 Flow Cytometry Method

Flow cytometry was performed with a Coulter Elite ESP (Coulter Corporation, Pty Ltd, Florida, Miami, USA) using a 488-nm line of an argon ion laser. Green fluorescence was collected by using a 550 nm dichroic and 525-nm band pass. Data were analysed using Elite Software® version 4.02.

8.2.5 NK Cell Cytotoxicity Assay

8.2.5.1 Cell Lines

Three tumour cell lines were selected as potential target cells for the NK cytotoxicity assay. K562 is an erythroleukaemic cell line derived from a chronic myelogenous leukaemia in blast crisis of human origin (Lozzio and Lozzio, 1979). Jurkat is a cell

line derived from acute T-cell leukemia; T lymphocyte of human origin (Weiss *et al.*, 1984). P815 is derived from a mastocytoma; or mast cell tumour of mouse origin (Ralph *et al.*, 1976).

8.2.5.2 Cell Culture Conditions

All cells selected were continuous cell lines and were cultured in Dulbecco's medium with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 90%; fetal calf serum, 10%. K562 was grown in T 25 flasks (Corning Glass Works, New York, US). P815 and Jurkat were cultured in T 75 flasks (Corning Glass Works, New York, US). All cell lines were cultured in a 5% CO^2 humidified air incubator. The temperature was maintained at a constant 37°C in a fully humidified atmosphere of 5% CO_2.

8.2.5.3 Cell Counts and Viability

Cell suspensions were diluted 1:1 with 0.25% Trypan blue in PBS. 10 µL of diluted cell suspension were transferred under the coverslip of an improved Neubauer haemocytometer counting chamber (Assistent, Germany). Using light microscopy the unstained (viable) and stained (non-viable) cells were counted to obtain the total number of viable cells per mL of aliquot. This count was used to determine the number of cells in each sample and the cell concentration calculated accordingly.

8.2.5.4 ^{51}Chromium-release Assay and Target cell Labelling

The NK cytolytic activity is usually determined by measuring the release of radiolabeled chromium from target cells that have been exposed to effector cells. The chromium-release assay permits quantification of cell mediated cytotoxicity. Target cells accumulate the isotope during incubation and cell viability is determined as a function of chromium release. Cells were harvested from frozen samples and maintained in cell culture flasks until confluent cell growth was achieved. Cells were pelleted ($1\text{-}2 \times 10^6$) target cells and resuspended in a small volume (<100 µL) of

medium. Cells were incubated with 100 μCi (3.1MBq) of $Na_2{}^{51}Cr_4$ (sodium chromate) (volume depends on specific activity ie. relates to the extent of radioactive decay) for between 1 and 1.5 hours in a 37°C water bath. Cells were centrifuged at 800 *g* for 5 minutes then washed twice in PBS, and once in Dulbecco's culture medium and finally resuspended in 1 mL culture media for cell count. Cell counts were performed and adjusted to a density of 10^5 cells/ mL.

8.2.5.5 Effector Cell Preparation

Peripheral blood mononuclear cell separation was performed from *P. gunnii* whole blood samples as described in Chapter 2; 2.8.1. The cells were counted and adjusted, where possible, to a concentration of 5 x 10^6 cells / mL in Dulbecco's culture medium.

8.2.5.6 Cytotoxicity Assay

One hundred μL of Dulbecco's cell culture medium was added to each well of a U-bottomed microculture plate (Greiner Labortechnik, Germany) and 200 μL of effector cell suspension was added to the first wells and doubling dilutions performed. The final volume of each well was 200 μL Effector cells (E) (100 μL) with a constant number of target cells (T) (100 μL). The resultant E:T ratios ranged form 50:1 to 3:1. Minimum target ^{51}Cr release was assessed by incubation of target cells with 100 μL Dulbecco's while maximal release was determined by treatment of the target cells with 100 μL 1% Triton X-100 in water (No. T6878; Sigma Chemical Company, St Louis, USA). The plate was then centrifuged at approximately 600 *g* for 4 minutes to facilitate the interactions. Cells were incubated at 37°C for 4 hours and 24 hours in a 5% CO_2/95% air incubator and the supernatant harvested and evaluated for γ-radiation evaluation using an automatic gamma counter (LKB Wallac 1277, Gammamaster, Turku, Finland). The percentage of cytotoxicity was calculated in relation to the spontaneous and maximal releases of target cells in medium and 0.1% Triton X-100, respectively as described by Whiteside (1991).

Percentage cytotoxicity was automatically calculated according to the formula:

$$\% \text{ cytotoxicity} = \frac{(\text{CPM experimental} - \text{CPM minimum}) \times 100}{(\text{CPM maximum} - \text{CPM minimum})}$$

- CPM experimental= counts from E:T ratios
- CPM minimum= counts from T + Dulbecco's
- CPM maximum = counts from T + Triton X-100

8.2.5.7 Nitroblue Tetrazolium Slide Test

This protocol was adapted from a simple method for assessing the ability of activated neutrophils to generate reactive products of oxygen as described by Clark and Nauseef, (1991). A 1 cm diameter circle was drawn on a glass coverslip using a wax pencil. One drop of phorbol myristate acetate (10 µg / mL, PMA, Sigma Chemical Company, St Louis, USA) was used as the stimulus and placed on the coverslip within the circle and let to dry at room temperature. One to 2 drops of non-anticoagulated blood was placed within the circle of the coverslip and incubated for 45 min at 37°C in high humidity to allow a clot to form. Using forceps, the clot was carefully peeled off the coverslip and gently rinsed with PBS. Two or 3 drops of commercial NBT (Sigma Chemical Company, St Louis, USA) was placed on the coverslip within the circle and incubated for 20 min at 37°C in high humidity. The coverslip was gently rinsed with PBS, fixed in 100% methanol for 1 min, then rinsed briefly with water. The cells were counterstained with a few drops of safranin for 10 min, rinsed with water, and air dried. The coverslip was examined by light microscopy at x400 (high dry) or x1000 under oil. The percentage of NBT-positive cells was counted in one hundred consecutive *P. gunnii* neutrophils.

8.2.6 Immunisation

Three *P. gunnii* adults were selected for immunisation as a source for the production of antibodies. Two New Zealand white rabbits were used to develop anti-bovine serum albumin using purified BSA as the immunogen. All animals were held in captivity and housed in the Central Animal House at the University of Tasmania.

Animals were housed individually and acclimatised to their environment receiving food and water *ad libitum*. Animals were bled prior to immunisation and the blood was collected into a Microtainer serum separator tube (Becton Dickinson & Co., Rutherford, New Jersey, USA) with a gel interface. Samples were centrifuged and serum stored at -70°C. This serum was used as a control to ensure antibody activity detected later was due to the immunisation.

Complete Freunds Adjuvant (F5881; Sigma Chemical Company, St Louis, USA) was used as the preferred adjuvant. One mL of BSA was mixed with 1 mL Complete Freunds Adjuvant solution and emulsified by repeatedly and vigorously drawing the mixture into a 2.5 mL syringe (with an all-plastic plunger) fitted with an 18 gauge needle, and squirting it out again. The container was continually chilled on a bed of ice to keep the mixture as close to 4°C as possible. Once the mixture appeared homogeneous and white, the integrity of the emulsion was tested. When a drop of the emulsion remained discrete on the surface of ice-water without spreading the emulsion was ready to inject. The syringe was then attached with a 21 gauge needle for injection and air bubbles removed. Each animal was injected intramuscularly. Adults received an initial dose of 2mg/ mL BSA for a total on day 0.

P. gunnii were bled 10 to 14 days following priming immunisation and a blood sample collected. Serum was collected as described above. For booster immunisations, Freund's incomplete adjuvant (F5506; Sigma Chemical Company, St Louis, USA) was used as the adjuvant for all subsequent immunisations. The first booster was administered 2 weeks after priming immunisation. Boosters were given at day 14, 28, 42, 57. Animal blood samples were collected and serum separated prior to each booster. Antibody levels were particularly low at all intervals so animals were given a further booster dose of BSA alum precipitate on day 98 and 112.

8.2.6.1 Serological Procedures.

Standard microtitre procedures were employed for all serologic determinations. Round bottom microtitre plates were used for all assays (Greiner Labortechnik, Germany). Haemagglutinating antibody was assayed using 0.05 mL of serial dilutions in equal volumes of saline. A 5% suspension of sheep red blood cells (SRBC) diluted in Phosphate buffered saline (PBS) and coated with BSA, was added in 0.025 mL volumes and the plates were incubated for 4 hours at room temperature. Serum complement was inactivated by incubation at 56°C for 30 minutes. Haemagglutination titres (HA) were recorded as reciprocals of the highest dilutions of antibody causing complete agglutination.

8.2.6.2 Haemagglutination Titres

Haemagglutination titration of serum was performed using sheep red blood cells (SRBC) coated with BSA. SRBC were washed three times in normal saline. 400 µg of BSA with 0.001g Chromic Chloride dissolved in 1 mL of saline was combined with a 5% SRBC suspension and stirred using a magnetic stirrer for 10 minutes. The suspension was centrifuged down at 1500 *g*, for 6 minutes. The pellet was resuspended and washed in PBS twice before being resuspended. Commercial anti-BSA antibody (Sigma Chemical Company, St Louis, USA) and anti-BSA developed in the New Zealand rabbits was used as controls.

8.3 RESULTS

8.3.1 Analysis of Phagocytosis using Light Microscopy

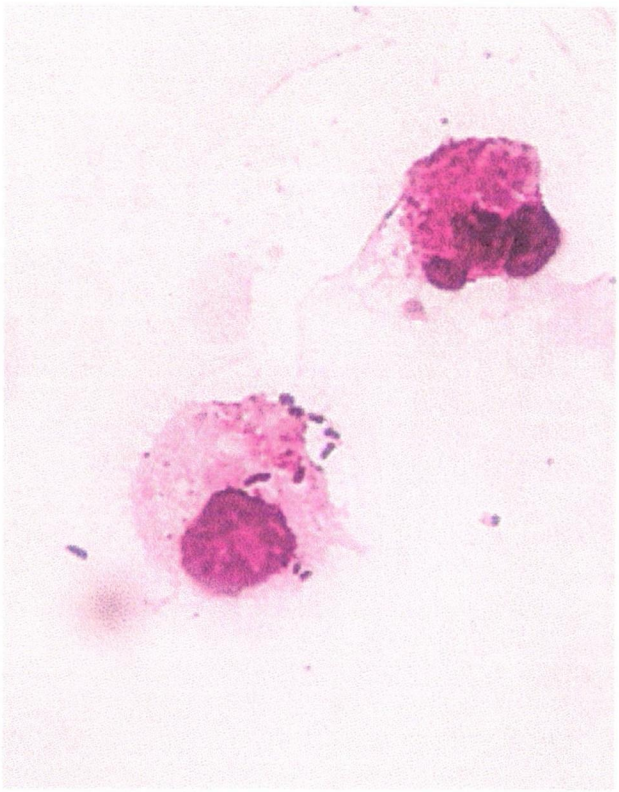
Peripheral blood from *P. gunnii* was incubated for 20 minutes with opsonised *E. coli*. A sample of cells was placed on a slide, dried and Gram stained. The smears were scanned to find Gram negative rods adhering to the neutrophils or with evidence of internalisation. Figure 8.1 demonstrates neutrophils with *E. coli* following 20 minute incubation and opsonisation as seen with ten *P. gunnii* samples processed. From

Figure 8.1

Gram stain *P. gunnii* phagocytic cells following opsonisation and 20 min incubation with FITC labelled *E. coli*.

Light microscopy

Magnification x1000



these preliminary results it was evident that neutrophils from *P. gunnii* have the ability to adhere and/or phagocytose bacteria.

8.3.2 Analysis of Phagocytosis using Flow Cytometry

Light microscopy provided evidence for effective phagocytosis. However, as it was difficult to quantitate the amount of phagocytosis and to distinguish between adhered bacteria and phagocytosed bacteria, a flow cytometry assay was developed. This assay was developed utilising heat killed *E. coli*, labelled with FITC and incubated with peripheral blood. The fraction of neutrophils that phagocytosed the fluorescently labelled bacteria was analysed and quantified. Trypan blue was used to quench the fluorescence if any FITC-labelled bacteria had adhered to the neutrophils and had not been internalised by phagocytosis. With this method, neutrophils that phagocytosed the bacteria could be quantified. The time for maximal phagocytosis has been shown to vary with neutrophils derived from a variety of clinical patient populations (White-Owen *et al.*, 1992), therefore, in regard to animal species, maximal phagocytosis times were established prior to each experiment.

To determine optimum time for the phagocytosis assay, peripheral blood was incubated with FITC-labelled bacteria and FITC-positive neutrophils were electronically gated and analysed for fluorescence intensity. Figure 8.2 typify the histograms obtained from experiments using *P. gunnii* neutrophils to establish maximum fluorescence intensity (FI) times. The histograms demonstrate the FI at incubation times 15, 20, 30 and 60 minute respectively. Maximum fluorescence intensity was achieved after 20 minute incubation and this time point was selected for all consecutive experiments.

Representative results shown in Figure 8.3 demonstrate forward scatter (FS) versus profile of the fluorescence intensity of peripheral blood neutrophils before and after quenching with Trypan blue. Analysis of phagocytosis for *P. gunnii* No. 1 revealed that 63.5 % of cells were detected in the positive quadrant 2, while after quenching

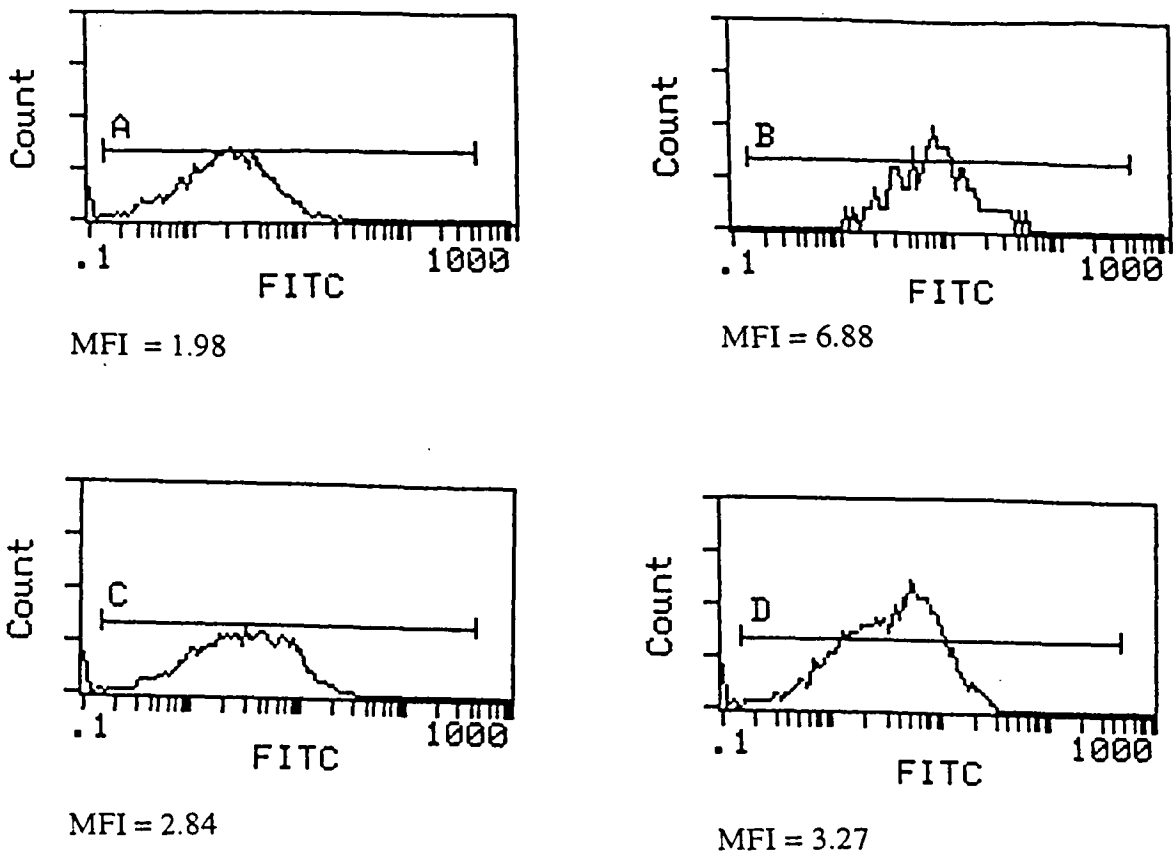


Figure 8.2

Histograms typify cell count vs relative FITC fluorescent intensity (FI) of *P. gunnii* neutrophils following phagocytosis at incubation times (A) 15 min, (B) 20 min, (C) 30 min, (D) 60 min. Maximum fluorescence intensity was achieved after 20 minute incubation and this time point was selected for all consecutive experiments.

Mean Fluorescent Intensity, (MFI).

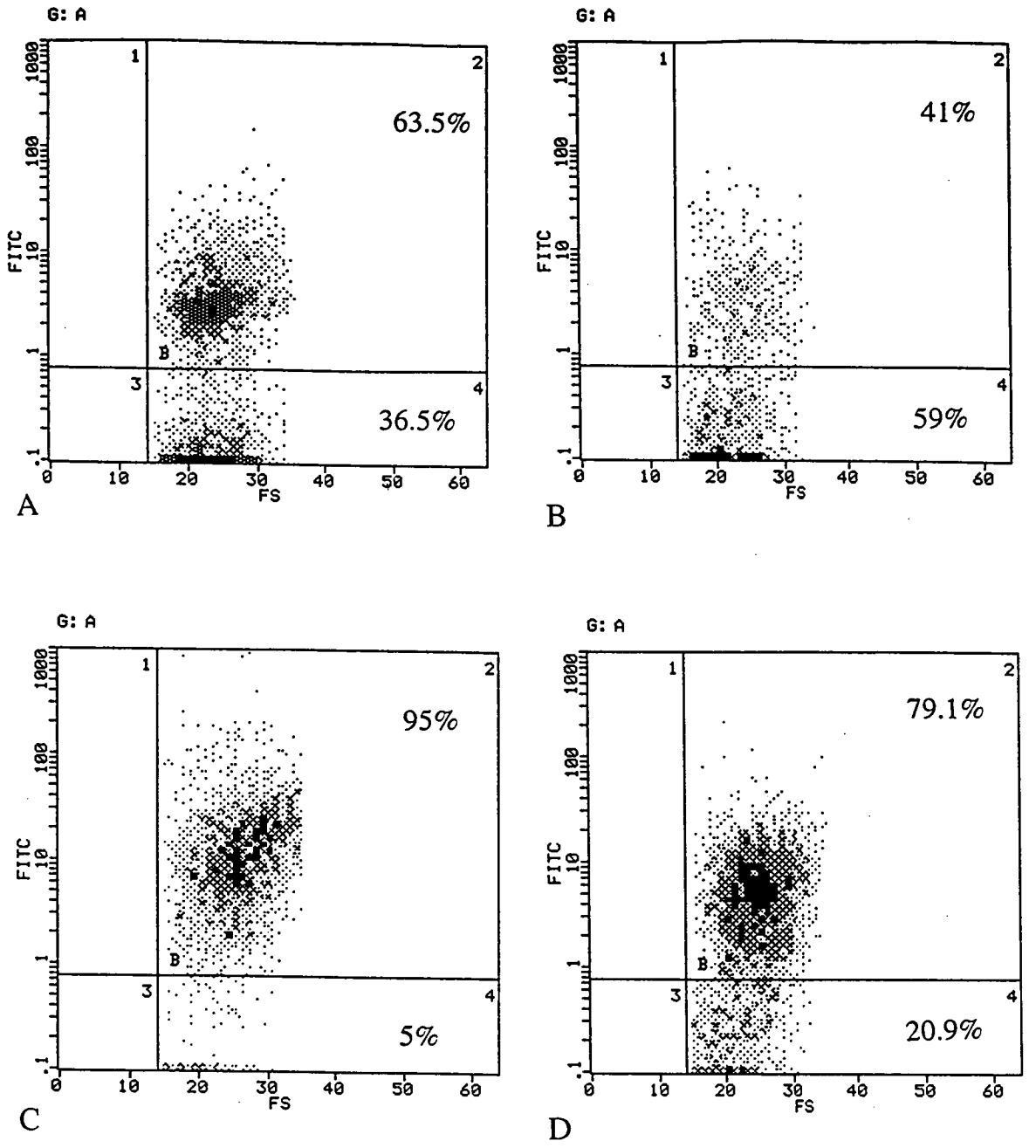


Figure 8.3 Dotplots represent percentage of neutrophils with FITC fluorescence after phagocytosis (A and C) and FITC fluorescence after quenching phagocytic cells with Trypan blue (B and D).

41% remained positive and this represented phagocytosis (Figure 8.3 A, B). *P. gunnii* No. 2, (Figure 8.3 C) revealed 95 % of cells detected were positive in quadrant 2, and after quenching 79.1 % remained positive, (Figure 8.3 D). Negative cells were quantified in quadrant 4. These results show that phagocytosis had occurred and this was consistently observed in all eight *P. gunnii* animals studied.

8.3.3 Confirmation of Phagocytosis using Confocal Laser Scanning Microscopy

Following flow cytometric analysis, a sample of cells was analysed to further provide evidence for phagocytosis using confocal laser scanning microscopy. Fluorescence images as shown in Figure 8.4, revealed that within 20 minute of incubation and opsonisation, neutrophils were detected with internalised fluorescent bacteria.

8.3.4 Nitroblue tetrazolium (NBT) assay

The conclusion for sections 8.3.1-8.3.3 was that peripheral blood neutrophils from *P. gunnii* possessed the ability to adhere to, and phagocytose opsonised bacteria. Consequently these initial stages of the phagocytosis process remains intact. The next step was to determine whether these neutrophils showed signs of activation that could lead to the digestion of the phagocytic material by using the NBT assay.

During phagocytosis, neutrophils exhibit an increase in oxidative metabolism and this activation can be detected by the nitroblue tetrazolium (NBT) reduction slide test which uses phorbol-myristate-acetate (PMA) as a stimulus. Reactive superoxide anion is produced as the first reduction product following activation and it is further reduced for reoxidation to oxygen. In its presence, formazan is produced and forms a blue-black precipitate and this is evidence for the activation of the oxidative pathway.

Peripheral blood neutrophils were stimulated by PMA and analysed for the presence of uniformly scattered blue crystals of formazan. Approximately. 95% (95 / 100 cells) of *P. gunnii* neutrophils were positive for NADPH oxidase activity using the

Figure 8.4

P. gunnii phagocytic cell following phagocytosis of FITC labelled *E. coli* using confocal microscopy. Note fluorescent bacteria inside cell and surrounding the nucleus.

Light Microscopy

Magnification x1000

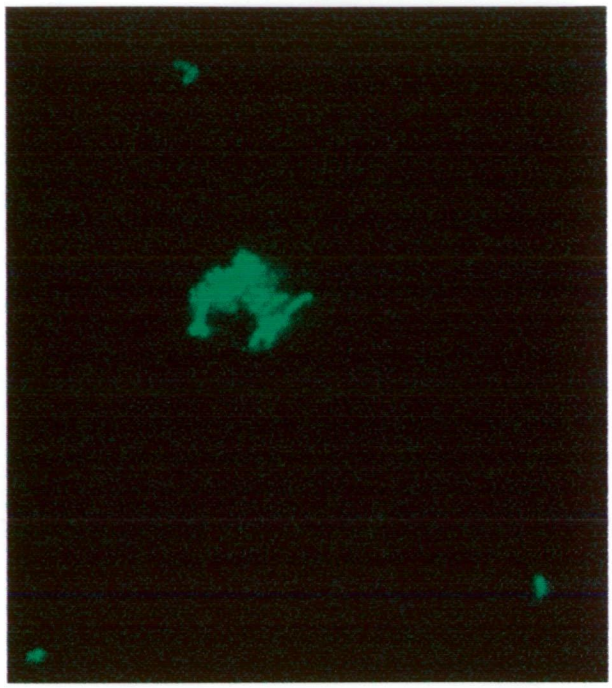
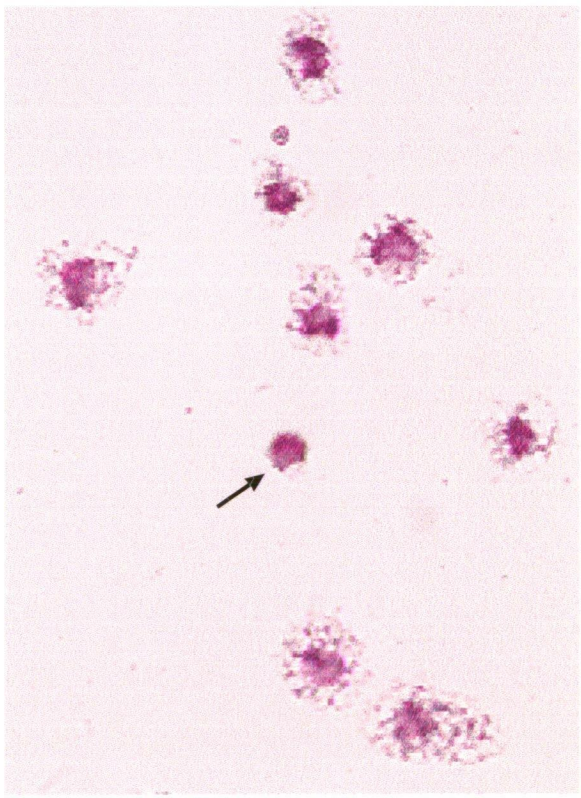


Figure 8.5

Photomicrograph of PMA-NBT positive stimulated phagocytes from *P. gunnii.* The normal NBT-positive cells appear large and degenerated whereas the negative (arrow) is vacuolised but intact.

Light Microscopy

Magnification x1000



simple slide test. Figure 8.5 is a representative photomicrograph of PMA-stimulated phagocytes from *P. gunnii*. Cells that reduce NBT and present with the blue formazan granules uniformly scattered over the cells appear large and degenerated, which indicates positive cells. Negative cells that have been activated but without concomitant reduction are vacuolized, but present well preserved with an unbroken cell membrane. These results suggest that *P. gunnii* neutrophils are capable of the oxidative pathway and therefore, have the potential to destroy microorganisms.

8.2.5 Measurement of Cytotoxic Activity of Natural Killer Cells

Natural killer cells (NK) are defined functionally by their ability to lyse target cells without prior sensitisation and without restriction by major histocompatibility (MHC) antigens (Robertson and Ritz, 1990). This cytolytic activity can be determined by measuring the release of radiolabeled chromium (^{51}Cr) from target cells, commonly tumour cell lines, that have been exposed to effector cells during a 4 hour incubation. The tumour cell lines Jurkat, K562 and P815 were selected as potential NK-sensitive targets as these cell lines are killed by human NK cells (Jurkat, K562) and mouse NK cells (P815). A range of effector cells to target cell (E : T) ratios were used to increase exposure of target cells to effector cells. The results revealed no significant ^{51}Cr release (Table 8.1). This suggested that cytotoxic activity by *P. gunnii* was not evident against these target cells. Similarly, the results of a 24-hr ^{51}C–release tumoricidal cytotoxicity assay exhibited no significant ^{51}Cr release.

8.3.6 Assessment of Mitogen Proliferation in Free-ranging and Captive Animals.

To assess the proliferative ability of *P. gunnii* lymphocytes, mitogen induced blast transformation was analysed. Two main groups of animals were selected. Group one comprised eight captive *P. gunnii* which were serologically negative for *T. gondii* antibodies. All captive animals were tested once a week for 3-4 weeks following capture to measure their responses during acclimatisation to their new surroundings. Group two comprised ten free-ranging *P. gunnii* selected from the capture and release program and contained two animals with detectable antibodies to

Animal	E : T	4 hours		24 hours	
		CPM	Specific cytotoxicity %	CPM	Specific cytotoxicity %
P. gunnii	JURKAT				
No. 1					
	50:1	86.6	5.4	568.8	19
	25:1	84.6	4.9	439.0	6.9
	12.5:1	63.0	0.1	444.0	7.4
	6.2:1	46.0	0	418.0	4.9
	3.1:1	75.9	2.9	364.5	-
No. 4	50:1	117.6	-		
	25:1	101.7	-		
	12.5:1	129.4	1.7		
	6.2:1	96.5	-		
	3.1:1	109.4	-		
		K562			
No.1	50:1	142.0	2.8	647.4	4.9
	25:1	145	3.1	580.9	1.5
	12.5:1	90	0	622.0	3.6
	6.2:1	82	0	680.0	6.6
	3.1:1	121.5	0.98	533.6	-
		P815			
No.2	50:1	179.4	2.7		
	25:1	189.1	4.8		
	12.5:1	206.4	8.6		
	6.2:1	201	7.4		
	3.1:1	-	-		
No. 3	50:1	176.3	7.2	704.0	12.9
	25:1	145.4	12.8	833.9	15.3
	12.5:1	169.15	8.9	-	-
	6.2:1	161.2	9.9	-	-
	3.1:1	-	-	-	-

Table 8.1 Cytotoxic activity of natural killer cells (NK), was determined by measuring the release of radiolabeled chromium ^{51}Cr from target cells (T), commonly tumour cell lines, exposed to effector cells (E) during a 4 h and 24 h incubation. A range of effector cell to target cell (E:T) ratios were used. The tumour cells used included Jurkat, K562 and P815.

T. gondii. A third group included animals experimentally infected with *T. gondii* oocysts, a component described in Chapter 3.

The data obtained for *P. gunnii* in captive and free-ranging animals were expressed as the stimulation index, in Figures 8.6 – 8.14. Each graph represents the incorporation of tritiated thymidine into DNA of *P. gunnii* blood leukocytes after culture with the mitogen. Each point represents the mean of triplicate or duplicate determinations on each animal. The stimulation index is defined as the relative incorporation of tritiated thymidine in the presence of mitogen compared to the incorporation of tritiated thymidine in the absence of mitogen. Counts were expressed as the Stimulation Index (SI) (CPM test sample/CPM medium control). Stimulation indices were used to allow standardisation or a comparison between different animals. Full data is presented in Appendix 11.8 -11.10.

Responses to T-cell mitogen Con A varied within the captive *P. gunnii* group. Maximum proliferative responses were recorded at Con A concentrations of 2.5 – 5 µg / mL (Figures 8.6 - 8.7). The maximum response was recorded for animal No. 1 at 230 SI, while poor responses (SI < 10) were recorded for animals No. 3, 4 and 5 at the same concentration. The highest proliferative responses obtained with Con A in free-ranging *P. gunnii* were at 1.25 - 5 µg/ mL. Best individual results were recorded for animals No. 15, 37, and 87 with maximum responses of 682, 169 and 366 SI, respectively.

Proliferative responses with PHA were variable amongst captive *P. gunnii* (Figures 8.8 – 8.9) with highest responses recorded at PHA concentrations of 1.25 – 5 µg / mL. Animals No. 4 and 7 recorded maximum responses of 463 and 294 SI, respectively. The lowest response at this concentration was recorded for animal No. 1. Overall responses for free-ranging *P. gunnii* were lower than those recorded for captive *P. gunnii* with responses below 10 SI. Maximum responses were recorded

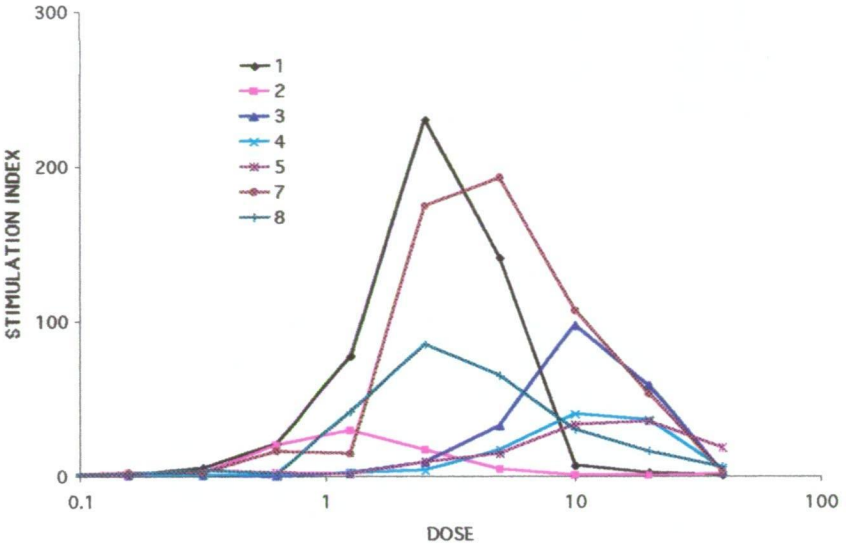


Figure 8.6. Proliferative responses of captive *P. gunnii* after culture stimulation with Con A mitogen.

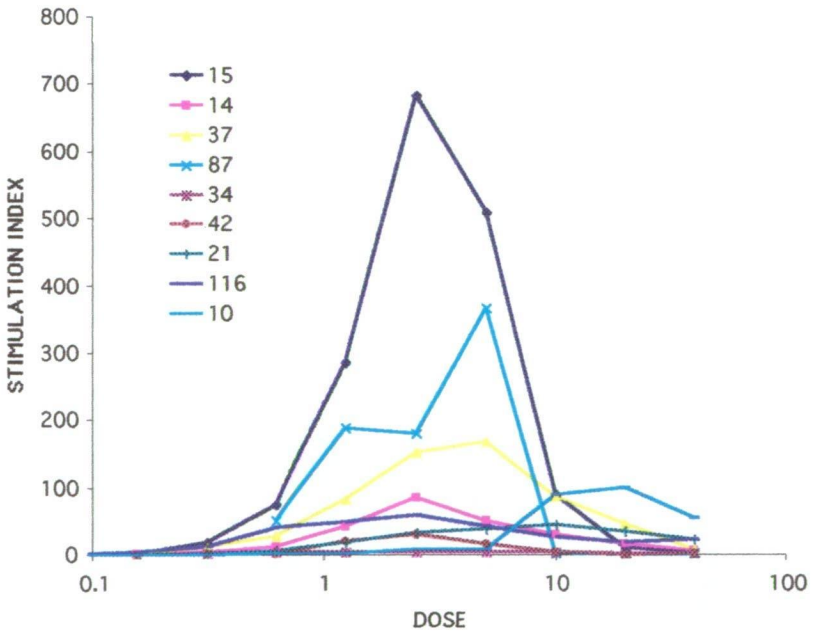


Figure 8.7. Proliferative responses of free-ranging *P. gunnii* after culture stimulation with Con A mitogen.

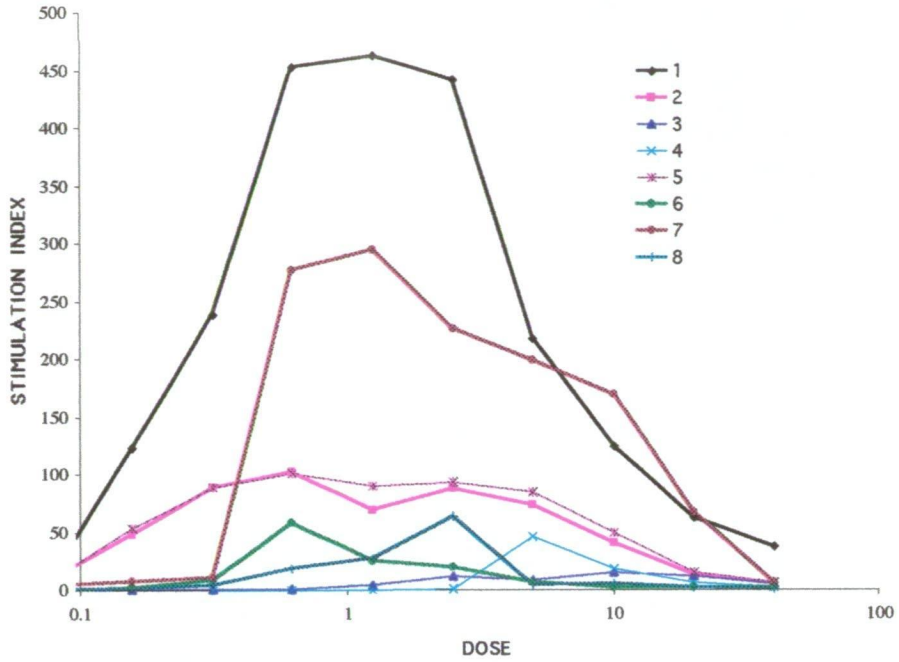


Figure 8.8. Proliferative responses of captive *P. gunnii* after culture stimulation with PHA mitogen.

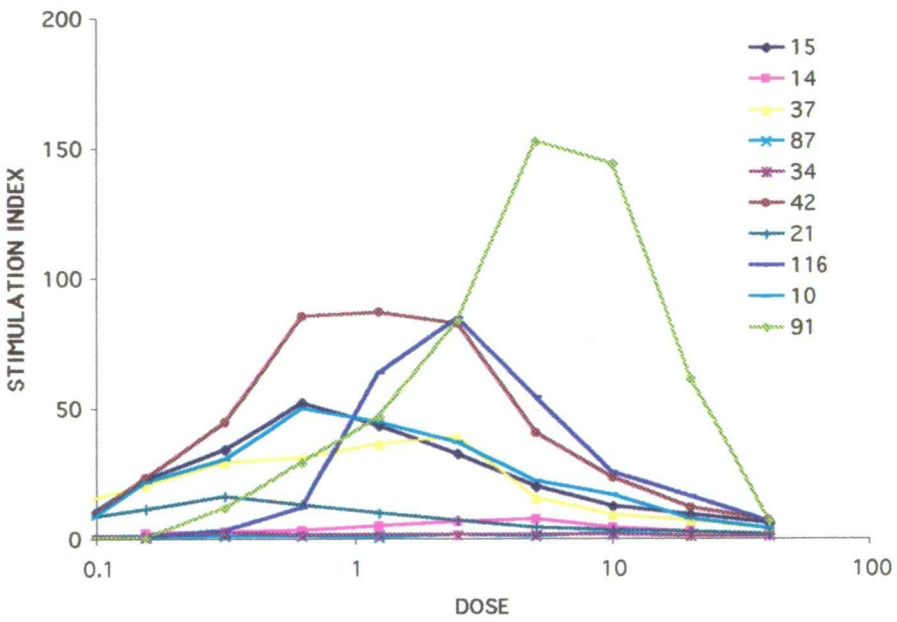


Figure 8.9. Proliferative responses of free-ranging *P. gunnii* after culture stimulation with PHA mitogen.

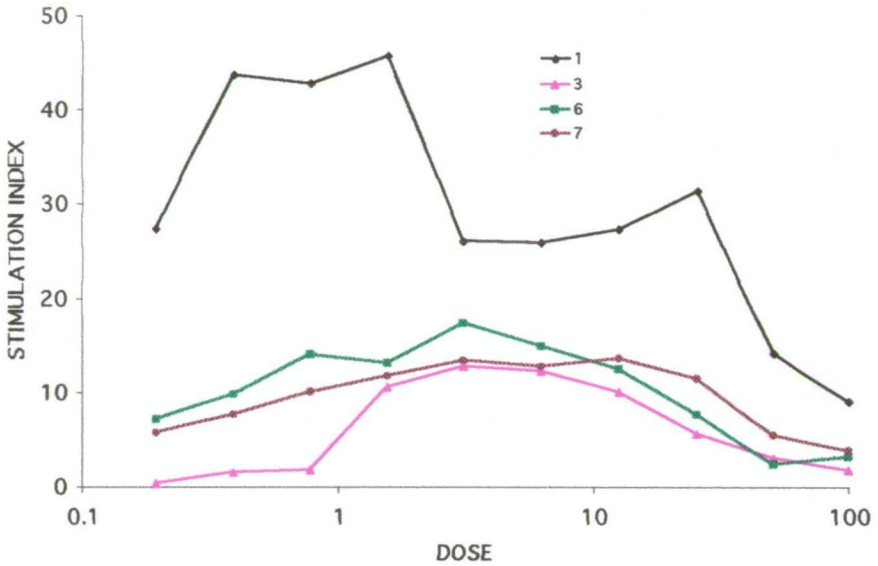


Figure 8.10. Proliferative responses of captive *P. gunnii* after culture stimulation with Pokeweed mitogen, (PWM).

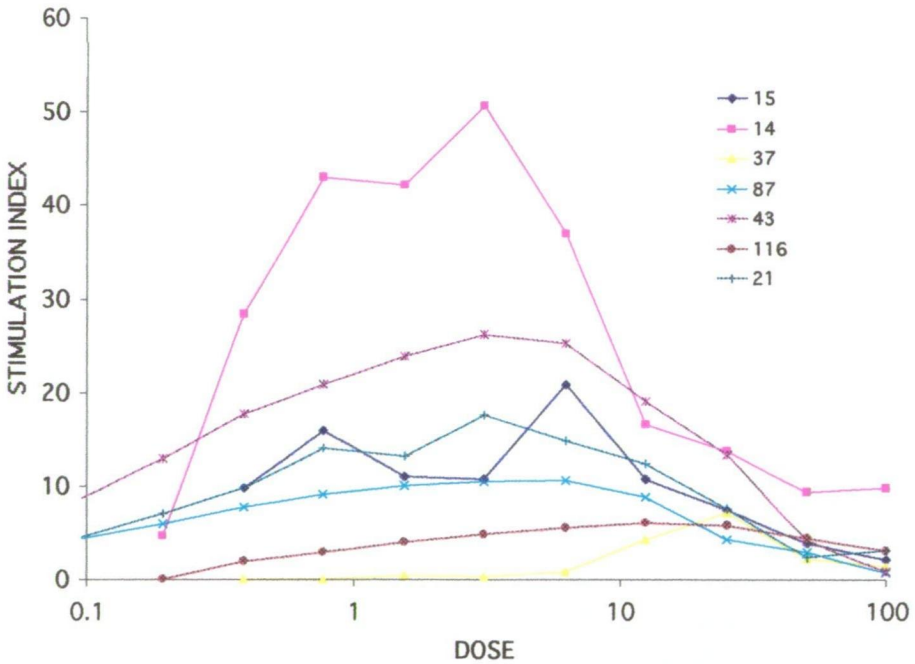


Figure 8.11. Proliferative responses of free-ranging *P. gunnii* after culture stimulation with Pokeweed mitogen (PWM).

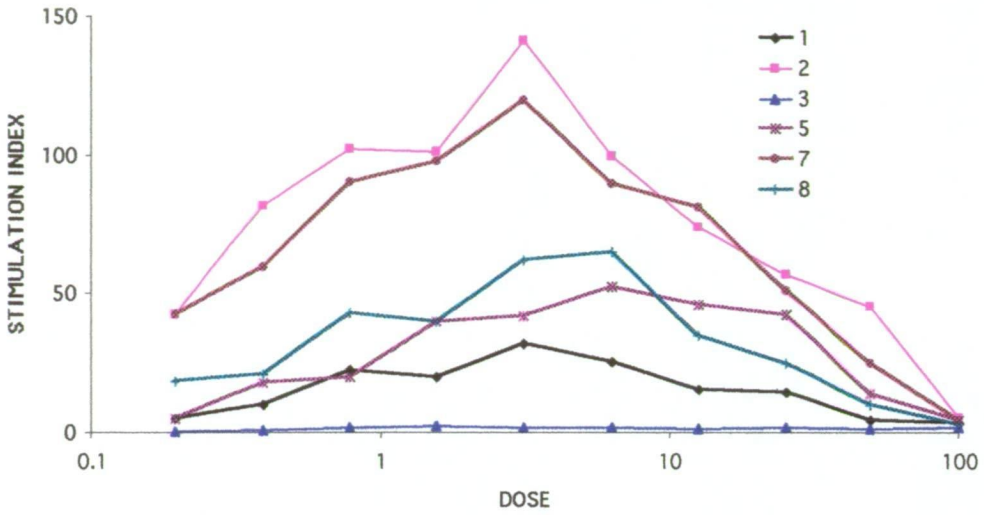


Figure 8.12. Proliferative responses of captive *P. gunnii* after culture stimulation with Jacalin mitogen (JAC).

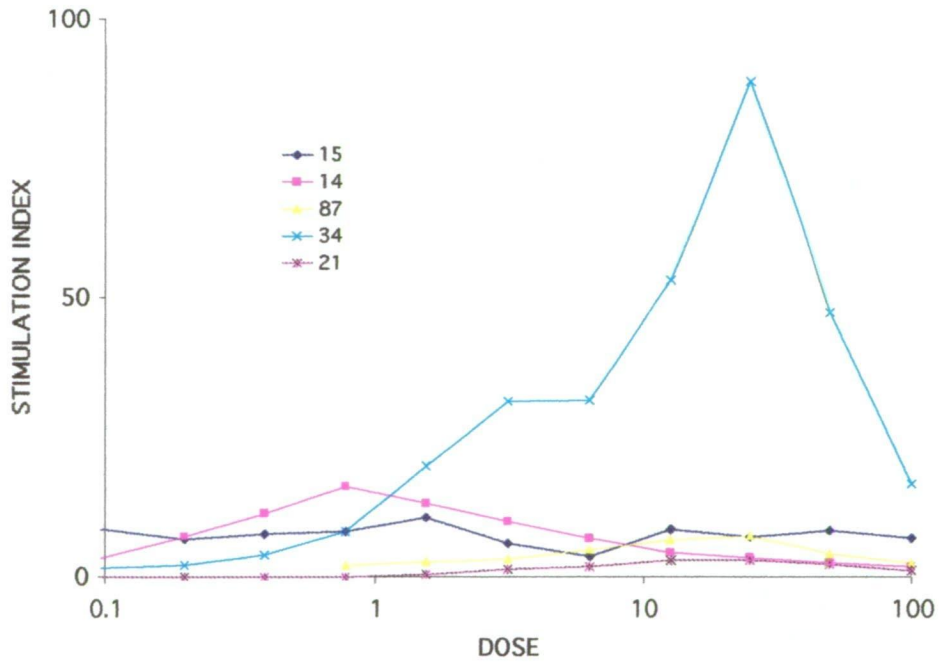


Figure 8.13. Proliferative responses of free-ranging *P. gunnii* after culture stimulation with Jacalin mitogen (JAC).

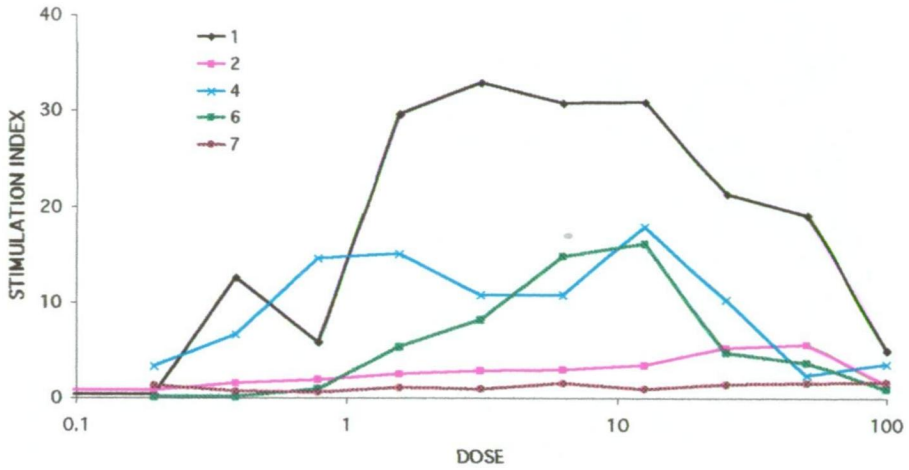


Figure 8.14. Proliferative responses of captive *P. gunnii* after culture stimulation with Lectin mitogen (LL).

for animals No. 43, 91 and 116. These ranged from 82 – 84 SI. Poorer responses were recorded for animals No. 14, 34, and 87 SI.

Proliferative responses of captive and free-ranging animals to T and B-cell mitogen Pokeweed mitogen were relatively low, with some animals responding below 10 SI (Figures 8.10 – 8.11). Peak responses for PWM were at concentrations of 0.78 – 6.25 µg/ mL. Responses to Lectin mitogen were relatively low with only two animal responses below 10 SI.

Proliferative responses of captive *P. gunnii* to B-cell mitogen JAC, were generally higher than free-ranging *P. gunnii* with responses exceeding 100 SI (Figure 8.13). Animal No. 2 and 7 recorded the highest proliferative response at JAC concentrations of 1.56-6.25 µg / mL. In free-ranging *P. gunnii,* only animal No. 34 responded well at similar concentrations recorded for captive *P. gunnii* (Figure 8.12).

Proliferative responses recorded for *T. gondii* antigen were poor with responses well below 5 SI in all *P. gunnii* tested (Table 8.2). Responses to Lipopolysaccharide (LPS) were relatively higher that *T. gondii* but variable with responses above 10 SI.

All animals in this study responded to at least one or more mitogens tested.

8.3.7 Immunisation of *P. gunnii* with BSA

To evaluate *in vivo* antibody responses of *P. gunnii* two animals were immunised with the T-cell dependent antigen, bovine serum albumin (BSA). Haemagglutination titres (HA) were recorded as reciprocals of the highest dilutions of antibody causing complete agglutination (Table 8.3). Booster shots produced only low responses. Booster injections of BSA in alum precipitate were administered with little if any change to titre. New Zealand white rabbits were used to developed anti-bovine

Stimulation Index (SI)										
Mitogen/ *P. gunnii*	**100 µg/mL**	**50**	**25**	**12.5**	**6.25**	**3.13**	**1.56**	**0.78**	**0.39**	**0.19**
TOXO 1	2.97	1.15	1.90	1.34	1.29	1.03	1.07	1.31	0.98	1.09
TOXO 2	2.59	2.39	1.98	2.29	2.13	1.39	1.75	0.63	0.63	0.28
TOXO 3	1.34	2.03	1.79	1.09	1.16	1.81	1.55	0.90	1.26	1.89
LPS 1	2.19	2.41	7.65	12.49	12.03	10.36	10.75	8.25	3.21	3.01
LPS 2	3.80	5.56	5.57	7.64	8.20	5.31	5.80	6.69	5.70	5.80
LPS 3	3.54	3.40	10.36	17.96	10.15	16.15	10.38	9.04	8.12	5.48

Table 8.2. Proliferative responses recorded for *T. gondii* antigen and Lipopolysaccharide (LPS) for animals No. 1, 2 and 3.

	P. gunnii		
Day Immunisation	No.2	No.15	Control
1	-	-	-
14	0:0	0:0	0:0
28	1:8	1:8	0:0
42	1:8	1:16	0:0
57	1:8	1:8	0:0
98	1:8	1:8	0:0
112	1:8	1:8	0:0

Table 8.3 To evaluate *in vivo* antibody responses of *P. gunnii,* two animals were immunised with the T-cell dependent antigen, bovine serum albumin (BSA). Booster shots were given on Day 14, 28, 42, 57. As antibody levels were particularly low animals were given a booster dose of BSA in alum precipitate on Day 98 and 112. New Zealand white rabbits were used to developed anti-bovine serum albumin and successfully produced a titre of 1:256 and 1:1024 respectively, providing evidence that the immunisation procedure was working. Haemagglutination titres (HA) were recorded as reciprocals of the highest dilutions of antibody causing complete agglutination

serum albumin and successfully produced a titre of 1:256 and 1:1024 respectively, providing evidence that the immunisation procedure was working.

8.2.8 Assessment of Immune Function of *T. gondii* Infected *P. gunnii*

Following the evaluation of responses to different mitogens and the observation of variability, amongst animals from the same population, the next step was to determine if infection with *T. gondii* could cause immunosuppression that would result in a reduced mitogen proliferative response. For this part of the project the animals analysed were exclusively used for experimental toxoplasmosis in Chapter 3, and were tested during the course of their infection.

Captive animals No. 01 and No. 03 were inoculated *per os.*, with *T. gondii* oocysts and No. 07 and No. 08 fed on worms infected with *T. gondii* oocysts as described in Chapter 3. Proliferative responses were analysed during the course of their infection. The initial proliferative assay was performed three weeks after capture when animals were acclimatised to their new housing facility. Lymphocyte proliferative assays were performed 8 days following infection for all animals except for No. 08. Full data is presented in Appendix 11.10.

Good proliferative responses Con A and PHA were recorded prior to experimentation (Figures 8.15 – 8.22). Responses to Pokeweed were poor, often below 10 SI but variable amongst individual *P. gunnii* (Figure 8.23 – 8.26).

Post-infection, all animals showed a sudden decline in response to T-cell and B-cell mitogens by Day 8. All mitogen responses were reduced following infection, with some values well below 10 SI. Toxo-infection progressed rapidly in each experimental case. No. 01 and No. 03 died 15 and 17 DPI, while No. 07 and No. 08 died 14 and 11 DPI. All animals remained seronegative for *T. gondii.*

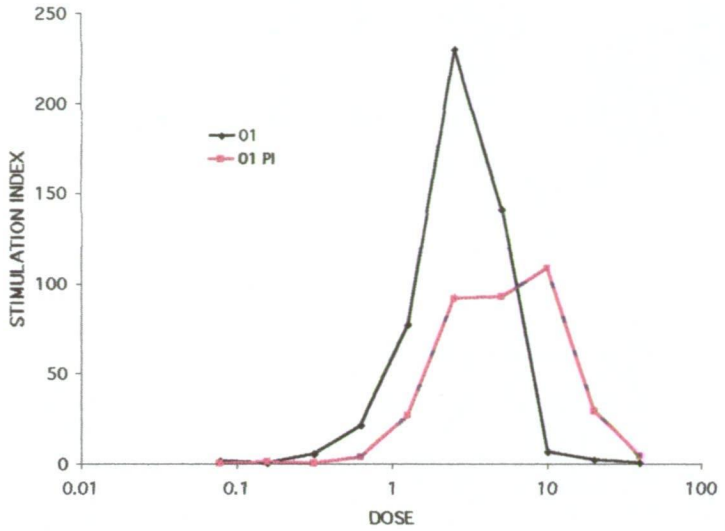


Figure 8.15. Proliferative responses of *P. gunnii* (01) leukocytes with Con A mitogen, pre and post-infection (PI) with *T. gondii*.

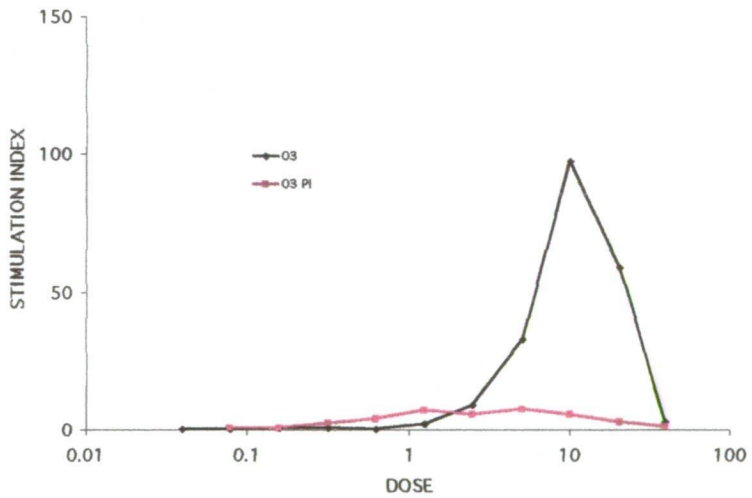


Figure 8.16. Proliferative responses of *P. gunnii* (03) leukocytes with Con A mitogen, pre and post-infection (PI) with *T. gondii*.

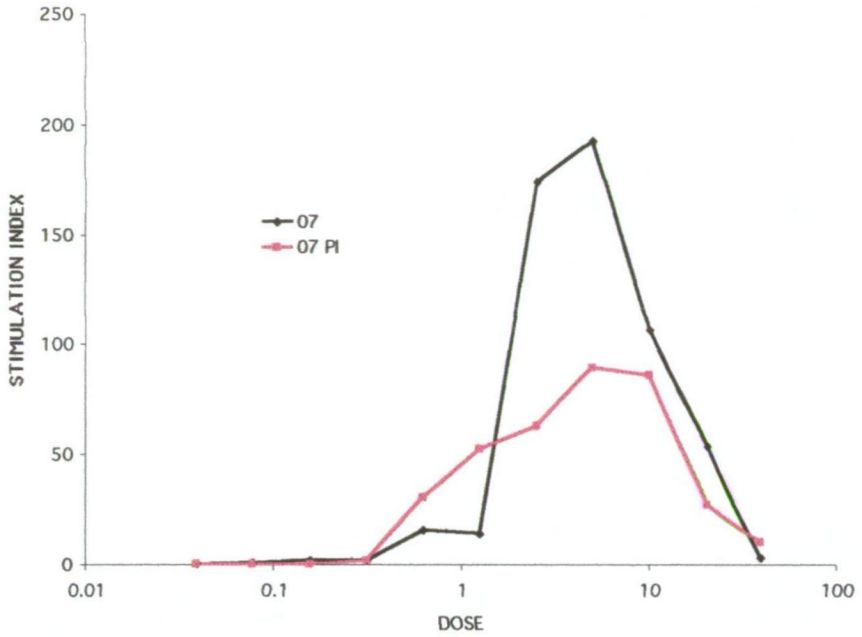


Figure 8.17. Proliferative responses of *P. gunnii* (07) leukocytes with Con A mitogen pre and post-infection (PI) with *T. gondii*

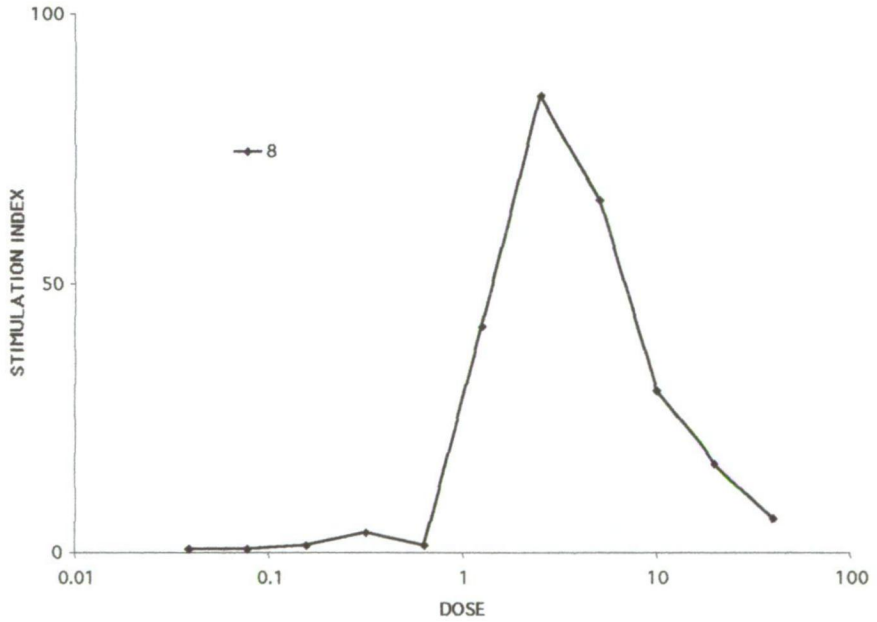


Figure 8.18. Proliferative responses of *P. gunnii* (08) leukocytes with Con A mitogen pre-infection with *T. gondii*

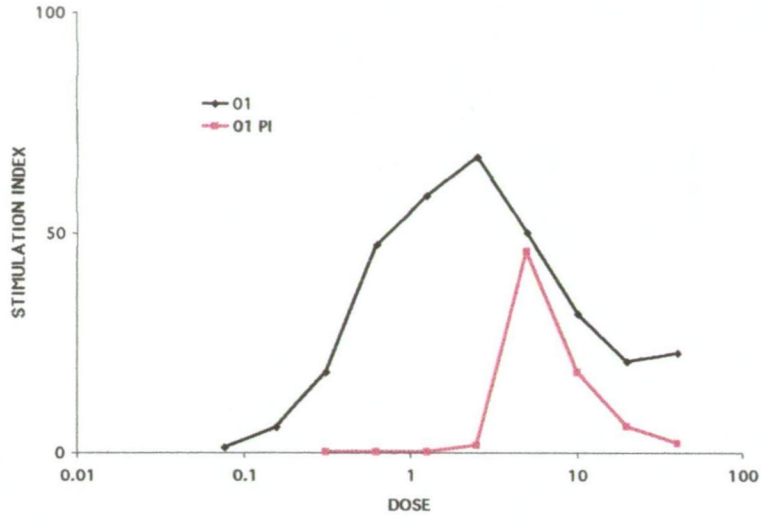


Figure 8.19. Proliferative responses of *P. gunnii* (01) leukocytes with PHA mitogen, pre and post-infection (PI) with *T. gondii*

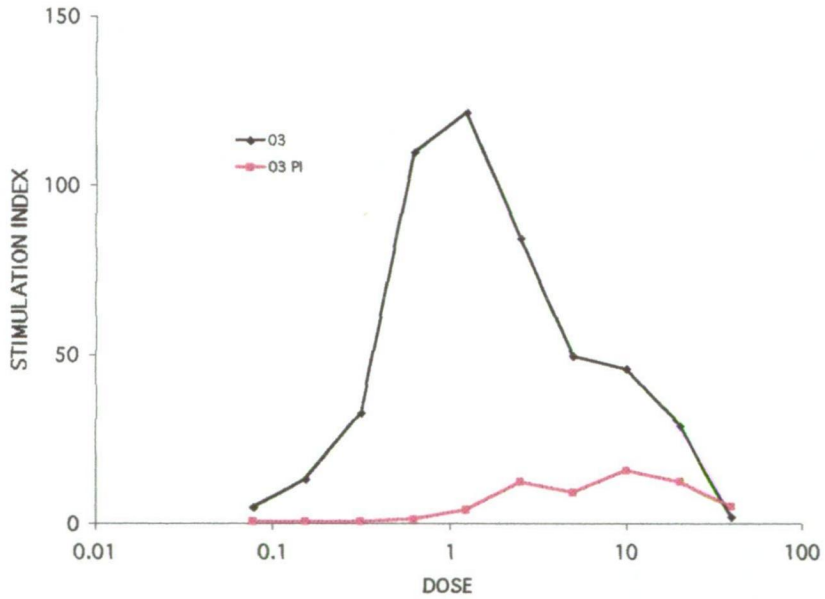


Figure 8.20. Proliferative responses of *P. gunnii* (03) leukocytes with PHA mitogen, pre and post-infection (PI) with *T. gondii*.

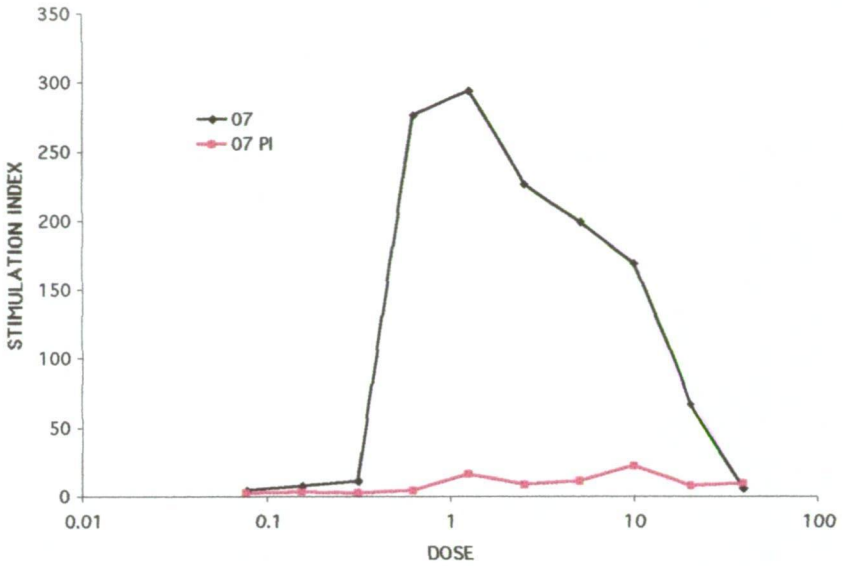


Figure 8.21. Proliferative responses of *P. gunnii* (07) leukocytes with PHA mitogen pre and post-infection (PI) with *T. gondii*

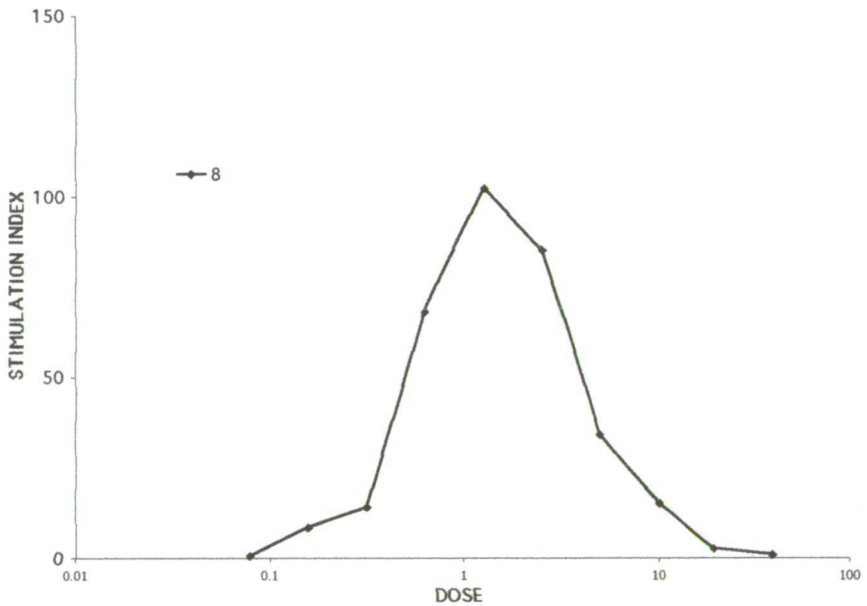


Figure 8.22. Proliferative responses of *P. gunnii* (08) leukocytes with PHA mitogen pre-infection with *T. gondii*

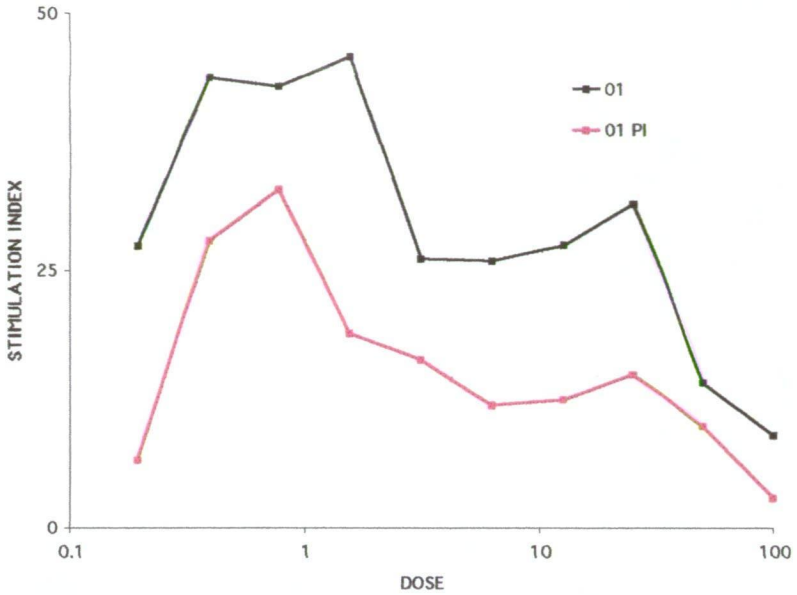


Figure 8.23. Proliferative responses of *P. gunnii* (01) leukocytes with PWM mitogen, pre and post-infection (PI) with *T. gondii*.

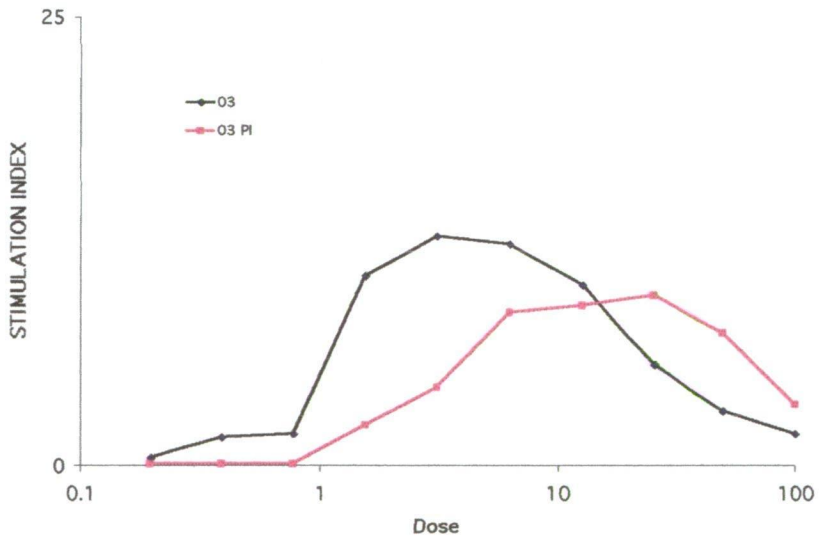


Figure 8.24. Proliferative responses of *P. gunnii* (03) leukocytes with PWM mitogen, pre and post-infection (PI) with *T. gondii*.

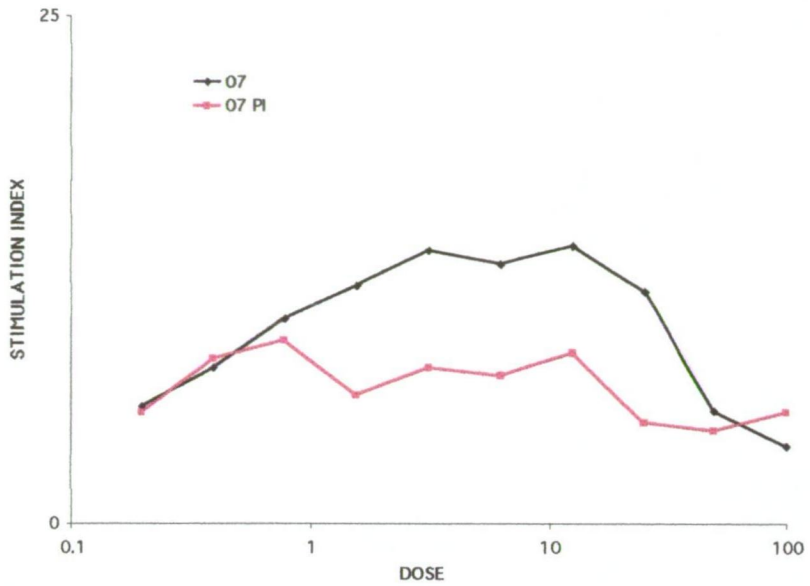


Figure 8.25. Proliferative responses of *P. gunnii* (07) leukocytes with PWM mitogen pre and post-infection (PI) with *T. gondii*

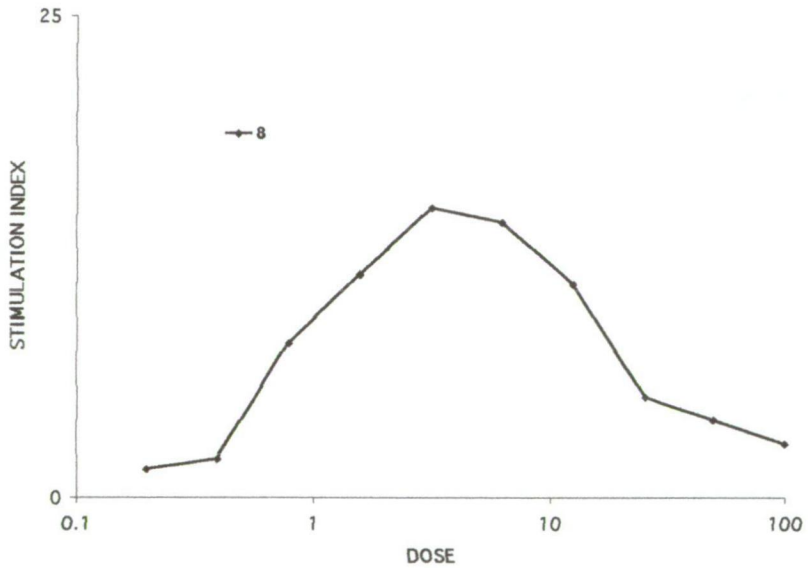


Figure 8.26. Proliferative responses of *P. gunnii* (08) leukocytes with PWM mitogen pre-infection with *T. gondii*

8.4 DISCUSSION

A failure to mount an adequate immune response may contribute to the susceptibility of marsupials to disease. Toxoplasmosis has been reported in wild bandicoots when often they show no clinical signs or significant antibody levels before their sudden death (Pope *et al.*, 1957; Obendorf *et al.*, 1990). Similarly, *in vivo* experiments with *P. gunnii* during the present investigation, have revealed that antibodies to *T. gondii* are not detectable in the terminal stages of the disease (Chapter 3). Clinical toxoplasmosis is much more likely to result if the immune capabilities of the host are diminished (Joynson, 1993; Dubey, 1994). Since responses to *T. gondii* are largely a function of both cell mediated and humoral immunity the susceptibility of marsupials to *T. gondii* infection suggests a defect in the arm of the immune response. Consequently, the cellular and humoral immune capabilities of *P. gunnii* were the focus of this chapter.

Phagocytic cells usually perform the early detection and rapid destruction of *T. gondii* tachyzoites. Tissue bound macrophages are the principal effector cells but to evaluate the capability of phagocytosis of the immune system, neutrophils were isolated from the peripheral blood of *P. gunnii* and their phagocytic response to microorganisms evaluated. Using an adapted whole blood assay and detection methods which included light microscopy, flow cytometry, and confocal laser scanning microscopy, it was apparent that neutrophils of *P. gunnii* were capable of initiating the first steps of phagocytosis, adherence and internalisation. Further work using the nitroblue tetrazolium assay indicated that these neutrophils were capable of activation that could lead to digestion of phagocytosed material. Consequently the major steps involved in phagocytosis appeared to be intact for neutrophils and it would be reasonable to assume that this was also the case for tissue macrophages.

Lymphocytes from many eutherian mammals respond to *in vitro* stimulation with mitogens, and this study showed that lymphocytes from free-ranging *P. gunnii*

behave in a similar manner. Given the paucity of comparative data, the results were difficult to interpret but it was generally confirmed that *P. gunnii* proliferative responses to mitogens compare reasonably well to similar investigations on other marsupials including koalas (*Phascolarctos cinereus*) (Wilkinson *et al.*, 1992) and Bennett's wallabies (Bourne, 1997).

Results of mitogen responses of *P. gunnii* in both captive and free-ranging groups revealed that T-cell mitogens such as Con A and PHA, have the highest and most consistent mitogenicity. Animals such as the koala, which are highly susceptible to *T. gondii*, show a good response to these T-cell mitogens, the best results detected being reported with Con-A (Wilkinson *et al.*, 1992). Responses by *P. gunnii* to PWM were not as high as the responses to PWM by the koala, which recorded an optimal response at a concentration of 50 µg/ mL. Both *P. gunnii* and the koala produce low responses in the presence of the T- and B-cell mitogen, Pokeweed mitogen. Responses in both animals were generally lower for B-cell mitogens including JAC and LPS. *P. gunnii* lymphocytes did not respond *in vitro* to *T. gondii* antigen. It has been recorded that seronegative marsupials such as Bennetts wallabies, respond poorly to *T. gondii* antigen (Bourne, 1997), so it was not surprising to find these results. Furthermore, the exquisite sensitivity of these animals to *T. gondii* may be a consequence of an inability of the immune system to respond to this pathogen. In reports on other marsupial species such as *Didelphis virginiana*, *Setonix brachyurus*, and *Macropus eugenii* PHA, Con A and PWM produce good lymphocyte proliferation responses (Ashman *et al.*, 1972; 1976; Keast and Bartholomaeus, 1972; Fox *et al.*, 1976; Ashman and Keast, 1977)

Often discrepancies amongst researchers and between investigations may result from different methods of blood preparation. Some have reported loss of monocytes in cell preparations that might account for some unresponsiveness to mitogens. Infante *et al.*, (1991) recorded the highest responses with Con A (maximal response at 25

μg/mL) from *Monodelphis domestica* but responses with PWM were weak and inconsistently mitogenic for this marsupial. Yet researchers such as Brozek *et al.*, (1992) recorded good responses with PWM in this species. A comparison of data reveals the discrepancy may be due to the fact Brozek *et al.*, (1992) used whole blood cultures, while Infante *et al.*, (1991) used mononuclear cell separation.

Results of free-ranging *P. gunnii* showed more variability in their responses than captive *P. gunnii.* It can be surmised that although care was taken to minimise noise and contact once animals were removed from the traps in the wild, the method of capture and release is a fairly stressful experience for the animal. Stress is known to increase the secretion of corticotropin-releasing factors, adrenocorticotropic hormone, glucocorticoides and β-endorphins (Mishra, *et al.*, 1994). It has been recognised that for some marsupials, handling stress and frequent blood sampling does affect the levels of hormones in the blood (Gemmell *et al.*, 1987). Work by Smith, (1992) found elevated corticosteroids in *P. gunni* during capture and handling, with the mean cortisol levels ranging from 5 pg/100 mL in males to 6.5 pg/100 mL in females and suggested that the first and most important requirement for experimental work with *P. gunnii* was the establishment of a period of acclimatisation in captivity.

The possibility that *T. gondii* interacts with the immune system to cause suppression was seen in animals infected with *T. gondii* with a significant response reduction to T-cell mitogens prior to death. This assay has successfully been utilised for several species of animals with clinical toxoplasmosis that may fail to develop measurable antibody titres, making diagnosis difficult (Yano *et al.*, 1983; Wilson *et al.*, 1990; Lappin *et al.*, 1992). This assay may have application in the diagnosis of toxoplasmosis in *P. gunnii,* when they fail to develop measurable antibody titres and clinical toxoplasmosis is suspected. Obviously there may be limits in application of this assay for free-ranging *P. gunnii* and possibly be restricted to animals in captivity,

or in a capture release program where repeat specimens and surveillance work can be performed.

Having seen that lymphocytes from *P. gunnii* responded poorly to *in vitro* stimulation with B-cell mitogens and that serology did not detect evidence for a humoral response in any of the animals infected with *T. gondii*, *in vivo* antibody responses in *P. gunnii* immunised with bovine serum albumin (BSA) were evaluated. The lack of antibody production from this immunisation program was consistent with the poor responses recorded with B-cell mitogens. This lack of ability, or what may essentially be a slow antibody production, suggests *P. gunnii* cannot activate the classical pathway of complement to lyse extracellular parasites, and without this ability, *P. gunnii* is extremely vulnerable to successful parasites such as *T. gondii.*

An attempt was made to determine the role of natural killer cells in *P. gunnii.* Although NK activity was originally described in the mouse and in humans, effectors resembling murine and human NK cells have been isolated in many vertebrate species (Robertson and Ritz, 1990). The poor results obtained for detection of cytotoxic activity of NK cells in *P. gunnii* with selected target cells, may suggest that NK cells if present are not providing these animals with early host immune protection against *T. gondii.* These results do not allow many conclusions to be drawn as several cytokines have been shown to enhance NK cell cytolytic activity including interferons (IFN-γ) and interleukin (IL-2) (Robertson and Ritz, 1990). A wider selection of target cells need to be incorporated into future investigations as well as methods of cytokine detection. It may be that the animals selected for this investigation were bled under stressful conditions as poor NK-cell activity in man and other animal species have often been attributed to stress (Konstantinova and Fuchs, 1990; Goulding and Flower, 1997). As *P. gunnii* succumb so early in the time course of toxoplasmosis, it may be that NK cells if present, are not playing a part in the early host immune response to *T. gondii.* Analysis of blood films

(Chapter 7) did not find evidence of large granule lymphocytes to which NK cells belong.

During the investigation a whole blood proliferative assay (Chapter 2; 2.9.4) was trialed as opposed to peripheral blood mononuclear cell (PBMC) separation from whole blood. Whole blood proliferative assays have often been introduced as an alternative to conventional methods of isolated lymphocytes by researchers (Paty and Hughes, 1972; Pellegrino *et al.*, 1973; Rella, 1978). The advantage is that small volumes of blood can be taken from small animals. The advantages lie with the technical simplicity and minimal alteration of cell elements by isolation procedures and processing. The major disadvantage is the variable content of lymphocytes in the individuals' blood. Unfortunately, this protocol could not be reproduced well with *P. gunnii* in the capture and release group. Seventeen *P. gunnii* were tested using whole blood assay but results were disappointing and inferior to the results obtained using mononuclear cell separation. Contamination was often a problem from samples taken by lancing the peripheral ear vein, a problem often encountered in field work. Clotting is a major problem when dealing with small volumes of blood. Blood-borne parasites were considered to a major problem in our population of bandicoots with interference of mitogen responses as the blood parasites survive in culture. It thus seems that screening of blood should be performed for every animal prior to experimental work.

This study highlighted an effective phagocytic pathway in *P. gunnii* cells and a capability of responding to T-cell mitogens thus suggesting a competency in cell mediated immunity. In contrast, the responses to B-cell mitogens and antigens were relatively poor and with a failure by *P. gunnii* to provide an antibody response when immunised with bovine serum albumin (BSA) it would appear that the humoral arm of the immune response is inefficient. As production of antibody is an integral factor in the function of opsonisation and consequent phagocytosis, then it is probable that

function of opsonisation and consequent phagocytosis, then it is probable that the susceptibility of *P. gunnii* to *T. gondii* can be accounted for by the poor antibody production, opsonisation and phagocytosis.

The experiments in this chapter have shown the potential in utilising standard laboratory techniques with slight modification for species such as *P. gunnii*. It has been possible to characterise the responses of PBMC to various mitogens and to show in the presence of diseases such as *T. gondii*, the suppression of cell proliferation. The methods outlined are reproducible and in a managed setting have future application in the management of vulnerable species. A recent report of the identification of T and B lymphocyte markers and antigen presenting-like molecules, in the Northern Brown Bandicoot as well as intra-cytoplasmic markers for CD3 and CD5 (Cisternas and Armati, 2000), demonstrates the potential for similar and more refined approach to investigate functional properties of immune system cells in *P. gunnii*. It is hoped that further progress and a range of prospective investigations will emerge from these results.

Chapter 9. Final Discussion

This thesis represents a study on the interactions of *Perameles gunnii* with factors that result in disease and mortality. The centre point of the thesis was the vulnerability of *P. gunnii* to infection with *Toxoplasma gondii* and from here the study was extended to consider the interactions with other parasites infecting this marsupial, the possible zoonotic potential between native animals and humans and the reservoir of inapparent toxoplasmosis. It explored the cellular and humoral responses of *P. gunnii* and their influence on the animal's response to *T. gondii*. It identifies the rich biodiversity and intricacies of parasite interactions with host and environment and highlights the on-going relationship between the host and such parasites as *Hepatozoon* and trypanosomes. It investigated details of the lifecycle of *Giardia* and potential transmission between wild animals and humans. It also indicates future lines of investigation, particularly as regards the newly identified blood parasites and *Giardia* spp., in the Tasmanian environment.

Although this work was not designed primarily as an ecological study, it does recognise that disease is one significant factor affecting native wild animal populations. It is well known that population stability fluctuates amongst many species in the wild due to factors such as predation, seasonal and climatic changes, and from the impact of environmental factors. However, one factor that is often overlooked is the influence of pathogens and their ability to influence long-term patterns in the cyclic abundance of animal populations.

A study of pathogenic organisms in any host species is important, not only for understanding the health of a particular animal but, for an understanding of factors which can influence the survival and perpetuation of the species in concert with ecological factors. Further, such studies can be important in relation to the sharing

of pathogenic or potentially pathogenic organisms with other animal species such as companion or domestic animals, and with humans themselves.

The recognition of the blood-borne parasite *Hepatozoon* is significant, as it may be an important predisposing factor rendering *P. gunnii* more susceptible to other diseases such as toxoplasmosis. This is the first report on the occurrence of the blood parasite *Hepatozoon* in *P. gunnii* (Bettiol *et al.*, 1996), with no other information presently available regarding the parasite's life-cycle, epidemiology, or pathogenicity. It is however, almost certainly transmitted by Ixodid ticks like the other members of the genus. There is little published material regarding the genus *Hepatozoon* itself, with much confusion existing over several of its species (Levine, 1982; Smith, personal communication). What role does the newly identified blood-borne parasite play in the survival of *P. gunnii*? The true significance of hepatozoonosis on the wild *P. gunnii* population remains to be determined as the animals observed in this investigation were held in captivity on a regular diet and without constant reinfection by the vector host and in the absence of other diseases. Electron microscope studies in the present investigation have, together with the fact that the protozoan infects erythrocytes, confirmed their apicomplexan nature and relationship to the genus *Hepatozoon*. As a direct result, many prospective investigations have emerged and the finding of the protozoan parasites will serve to further increase our knowledge and stimulate research into this and other species of marsupial haematozoa.

This study identified the first record of trypanosomes from *P. gunnii* (Bettiol *et al.*, 1998). The morphological features and dimensions recorded for these trypanosomes during the collaborative work with Lê, (1996), suggest that trypanosomes from *P. gunnii,* and those observed in the Southern Brown Bandicoot, *I. obesulus*, belong to a new taxon. The present study suggests that these trypanosomes are of low pathogenicity, but bearing in mind the endangered status of

P. gunnii, future studies are imperative to assess in detail what role these and other parasites may have in relation to the survival of this host species. Collaborative work has commenced using DNA analysis, with the primary objective to establish the identification of the species in Tasmanian bandicoots in comparison to the species identified as *Trypanosoma thylacis* by Mackerras (1959a) and in other species of bandicoots from mainland Australia. The study has opened up an exciting prospect for elucidating the life cycle of this species and determining its vector, which might be a flea. This would serve to enhance existing information regarding the species of marsupial haematozoa in general, and may allow control of infection through vector control, if required.

The detection and isolation of intestinal helminths of *P. gunnii* has confirmed and expanded the findings of other workers. The helminth parasites detected in *P. gunnii* were diverse and dominated by representatives of the Nematoda. Most previous studies merely presented check lists of the helminth species found, whereas this study has shown low helminth loads to be the rule, which is not surprising considering the solitary habits of *P. gunnii,* and thus not likely to cause much clinical disease. However, in captivity, crowding may cause load build-up and must be considered in future management plans.

The bandicoot is often noted as a potential reservoir for bacterial zoonoses and it has often been reported to play a role in contributing to the high incidence of human salmonellosis in Tasmania (Ball, 1991; Davies, 1995a). Although salmonellae were not detected in *P. gunnii* from this study, there is a strong possibility that contaminated faeces in wilderness areas and faeces from domestic stock in rural farming areas might act as a source of infection for humans. Other potential bacterial enteric pathogens were isolated which may impact on *P. gunnii* during times of nutritional deprivation, injury, concurrent disease or environmental stresses, such as severe winters, hot dry summers, captivity.

During this study it was possible to detect viral antibodies in *P. gunnii.* The presence of antibodies to Ross River (RR) virus suggests that *P. gunnii* are an important potential reservoir of RR virus. The effects of the RR virus on *P. gunnii* and other bandicoots are at present unknown given their occurrence in coastal areas of eastern Australia, northern Queensland and Tasmania. The fact that they can become infected with this virus indicates that they, with other marsupials, serve as a reservoir for the infection, which can be transmitted to humans via the mosquito vector.

With movement of human and animal populations, and as local and international trade in animal products increases, so does the risk of zoonotic diseases (Stohr and Meslin, 1997). There is the risk that such diseases may be introduced or reintroduced into certain areas. Evidence for this has occurred worldwide, an example being North America, with concern being expressed regarding the waterborne transmission of *Toxoplasma* and several species of microsporidia of public health significance (Bowie *et al.*, 1997; Dowd *et al.*, 1998). So too, *Leishmania* and *Trypanosoma cruzi* have extended their range into areas of Europe and the USA respectively, not previously regarded as endemic (Eckert, 1997; Meurs *et al.*, 1998). In Australia, dogs and cats have been detected to harbour genotypes of *Giardia* that are infective to humans (Hopkins *et al.*, 1997). Australia's capital cities in recent years have experienced the pressure of public health concerns with the detection of *Giardia* and *Cryptosporidium* in drinking water (Wade and Fairley, 1998). In Tasmania, changing agricultural practices, urban development and increasing tourism all offer opportunities for change in the distribution of zoonotic diseases and transmission patterns to occur. As wilderness areas are opened to more nature enthusiasts, so too is the increased risk of zoonoses. The impact of humans in these ecosystems is only now coming to the surface and being acknowledged. More must be learnt about these diseases, especially in native wild animals – the

species of pathogens, their epidemiology, transmission, effects on the natural animal host and their potential to infect humans.

The detection in this study of *Giardia* and *Cryptosporidium* from several of Tasmania's most isolated waterways has opened the challenge for the detection and identification of new genotypes and strains. Prospective investigations may implement the use of new, and perhaps more sensitive, detection methods such as PCR and tests to detect cyst and oocyst viability (Dowd 1998; Kaucner and Stinear, 1998; Mahbubani *et al.*, 1998). There is strong evidence that cross-infection with *Giardia* between native animals and humans can occur and has been confirmed in this present study (Bettiol *et al.*, 1997). *P. gunnii* may well play a part in the transmission of *Giardia* which has been deemed to be responsible for outbreaks of diarrhoea in bushwalkers (Kettlewell *et al.*, 1998). Similarly, the impact of bushwalkers and visitors and their movements is of equal concern, especially in their role of parasite distribution and transmission to other animals.

In terms of clinical affects on *P. gunnii*, and also of zoonotic potential, infection with *T. gondii* is of particular significance. *P. gunnii*'s rapid death and lack of detectible antibodies from experimental inoculation of *T. gondii* oocysts demonstrates their vulnerability to toxo-infection (Bettiol *et al.*, 2000a). Since resistance to *T. gondii* is largely a function of cell mediated immunity, the susceptibility of *P. gunnii* to toxoplasmosis suggested a defect in this arm of their immune response. The practicality of measuring cell-mediated immunity in *P. gunnii* was demonstrated in a series of experiments using *P. gunnii* peripheral blood mononuclear cells and standard laboratory techniques. Modifications of available techniques were required in many instances and obvious problems arose due to the difficulties in using native wild animals in a capture and release program. The experiments have shown that *P. gunnii* leukocytes are capable of phagocytosis. Most individual animals that were tested responded to stimulation with T-cell

mitogens but responses to B-cell mitogens were low and variable. Responses of animals infected with *T. gondii* demonstrated a decrease in responses to all mitogens selected suggesting that infection with *T. gondii* leads to suppression of T-cell mediated immunity. Humoral responses were particularly poor with the immunisation protocol used, suggesting these animals have a slow response time. It remained inconclusive whether these animals have natural killer-like cells. As *P. gunnii* succumb rapidly to toxoplasmosis as described in the experimental studies, it may well be that natural killer (NK) cells are absent as they do not appear to slow down the infection.

The vulnerability of *P. gunnii* to toxoplasmosis emphasises the fact that *Toxoplasma gondii* is a highly successful parasite in terms of geographic spread, susceptible host range and transmission. The knowledge that the parasite completes its sexual cycle only in cats indicates possibilities for prevention strategies for the disease but apart from avoidance or prudence, no prophylatic measures are presently available.

Cats can acquire infection by hunting wild mice and birds, eating raw meat or via the faecal-oral route by ingestion of oocysts passed by other cats. Herbivores including livestock become infected by ingestion of grass and other foodstuffs that have been contaminated by cat faeces. Feral or farm cats are potential sources of *T. gondii* but, which of these is the more important is not known. Young cats appear to be the major source of oocysts as large numbers are produced for 1-3 weeks following initial infection because a high level of resistance is soon established (Dubey and Frenkel, 1972; Dubey and Beattie, 1988). Although older cats may re-shed small numbers of oocysts, this is thought to occur infrequently (Dubey and Frenkel 1976; Dubey,1986). Rapid and uncontrolled breeding of feral cats, with a constant supply of new-born kittens could well be a significant factor for *Toxoplasma* transmission in feral cat populations.

Feral cats are widespread throughout Tasmania and are a prominent feature of the landscape, having reached plague proportions in some areas (Driessen, personal communication). In many instances, farm cats are likely to outnumber feral cats on a farm and may well be the more important source of *T. gondii* infection. In New Zealand, it was determined that about 80% of farm cats were reliant on predation and scavenging for survival (Charleston, 1994). All were likely to be infected by ingesting tissue cysts in rodents, rabbits, and other meats. Animals such as rabbits are known carriers of *T. gondii* (Munday, 1970) and are found throughout the farming areas of Tasmania. Cats are capable of consuming the entire carcass of such prey, but if *P. gunnii* becomes their prey, the cat will only consume the head of the marsupial, leaving the rest of the carcass behind (Goldsmid, personal communication). This opportunistic behaviour increases the risk of exposure to *T. gondii,* as tissue cysts are found especially in the central nervous system (Charleston, 1994).

From the perspective of domestic animals, *Toxoplasma gondii,* has long been recognised as a parasite of considerable importance to the Tasmanian and New Zealand sheep industry. Work by Munday, (1972b) and Hartley and Munday, (1974) showed a relationship between *T. gondii* infection in sheep and contact with cats. Toxoplasmosis remains one of the most common infectious agents involved in abortions, stillbirths and neonatal deaths in sheep, with infection rates second only to *Campylobacter* (Charleston, 1994). Abortions due to toxoplasmosis are sporadic and unpredictable, both in occurrence and scale and can be determined by the proportion of the flock that is not already immune due to previous infections and those that were exposed during pregnancy (Charleston, 1994). For the farmer this can be devastating due to economic loss, magnified by lower wool production and the loss of both ewe and their lambs, not to mention the extra managerial costs with neonatal deaths and intermittent abortion storms.

Many farmers consider that preventing cats from becoming infected would be impossible and perhaps even undesirable, since infection of sheep before pregnancy is protective for the foetus. Veterinarians often recommend that, if abortions occur in an area or paddock the previous season, it is advisable to move all stock onto that site with the aim of exposing the animals to *T. gondii* before pregnancy (DPIWE, personal communication). From a public health point of view this is undesirable as sheep meat is a known potential source of human infection as *T. gondii* organisms can be isolated from muscle in a high proportion of sheep with antibodies to *T. gondii* (Munday, 1975; O'Donoghue *et al*, 1987).

In many instances marsupials such as pademelons and Bennett's wallabies frequently graze in the same area as sheep and in both species the carrier rate of *T. gondii* is often very high (Munday, 1970). Bennett's wallabies in particular, are susceptible to toxoplasmosis (Bourne, 1997). From a public health perspective, changes of food preparation and eating habits by humans in recent times have seen an increase in consumption of game meats such as wallaby and kangaroo – often eaten undercooked to preserve tenderness. Consumption of exotic meats can also lead to human foodborne outbreaks of toxoplasmosis as recently suggested in an outbreak in the state of Queensland after ingestion of kangaroo meat (Robson *et al.*, 1995).

Recently many Tasmanian farmers, for financial reasons, have taken on the new trend of subdividing land for property development. With increasing incidences of toxoplasmosis in domestic stock often being reported on farmland bordering these developed areas, it has been suggested that farmland, once isolated, may now be exposed to domestic cats that wander onto neighbouring properties (DPIWE, personal communication). The problem is of growing concern for the Department of Parks and Wildlife, as natural reserves also border onto subdivisions and farmland, increasing the risk of toxoplasmosis amongst the native marsupial

populations. As detailed in Chapter one, *P. gunnii* often move into suburban areas as low shrubs and undercover persist on bordering properties, offering them shelter and nesting sites. Due to habitat changes bandicoots have thus wandered into dangerous areas, exposing themselves to the competitive and predatory nature of feral and domestic cats and to disease transmitted by these predators.

P. gunnii appear to have little protection from a previous infection or experimental challenge with *T. gondii.* In this they differ from sheep, in which serological evidence of a previous infection protects them against natural infection (Dubey and Towle, (1986) cited by Thompson, 1999) and also has substantial protection in pregnant animals, if experimentally challenged (Jacobs *et al.*, 1961; Jacobs and Hartley, 1964). The interaction of *P. gunnii* with domestic animals in many rural areas of Tasmania is high. At the same time it is logical to speculate a continuous and complex cycle established amongst farm stock and humans as well as farm stock and wildlife (Figure 9.1) - not only with bandicoots but also with macropods, including wallaby and possum, as they too have been reported with high rates of *T. gondii* infection. We see both herbivores and omnivores infected, which leads to speculation that soils contaminated with *T. gondii* oocysts from cat faeces are a source of infection. Oocysts may contaminate the vegetation while earthworms were proven in this present study to be a good paratenic host (Bettiol *et al.*, 2000b). The growing practice amongst landowners to restock pastures with earthworms to improve soil fertility, provides a wider source of contamination and perpetuation of *T. gondii* to pasture land in Tasmania. The earthworm seeding of pastures should then only be undertaken after a feral cat extermination programme has been instituted for the area.

In addition to the problem of toxoplasmosis endangering *P. gunnii* populations, the growing pressures on *P. gunnii* resulting from habitat destruction, the presence of humans and, domestic and companion animals is certainly an issue that requires

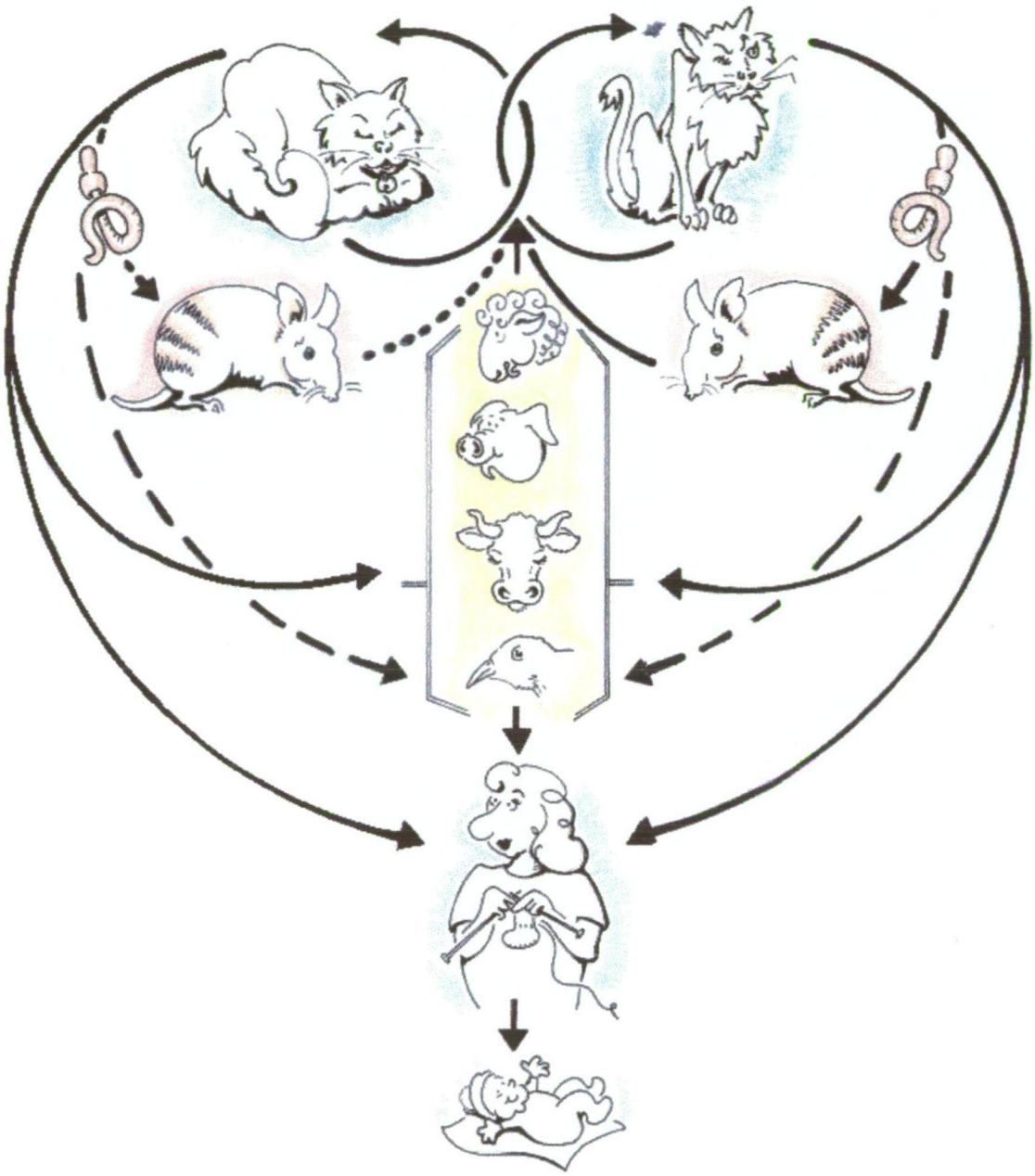


Figure 9.1 The epidemiology of toxoplasmosis in Tasmania. Intermediate hosts are infected by the ingestion of *T. gondii* oocysts with direct contact from both domestic and feral cats (solid arrow). Native *P. gunnii* are more likely to be infected by direct contact with feral cats and by the ingestion of earthworms. Infection in domestic and wild animals can occur by the ingestion of oocysts transported by invertebrates such as the earthworm (broken arrow).

attention. Municipal councils need to be more stringent in their process of evaluation and implementation of legislation and approval of rezoning agricultural land into residential areas, especially land that may border onto Crown Land or natural reserves.

Parks and Wildlife have realised that members of the community have a fervent interest in conserving threatened species. The Threatened Species Strategy and Natural Heritage Trust were established with this purpose in mind. Landowners have been invited to become more involved in conserving threatened species on their properties and several land management agreements are being developed between the Parks and Wildlife Service and landholders in Tasmania. The Threatened Species Program participates in protecting the habitat, which is complemented by programs such as Bushcare and the Regional Forest Agreement Private Land Reserve Program (Personal communication; Parks and Wildlife Service; DPIWE).

Recognition of disease agents in individual animals is an important starting point for disease surveillance of native wildlife, a point reinforced by veterinarians and conservationists alike (Obendorf, 1995). The scope of veterinary and medical parasitology has had to encompass wildlife because of its role as a potential reservoir of parasitic infections, and the impact these diseases have upon conservation of native fauna (Thompson, 1999). More importantly these pathogens can induce many and varied expressions of disease in the animal besides mortality. Obendorf, (1995) described such expressions of disease to be observed in individual animals making up a population and yet not jeopardising the stability of the overall population. Certainly one growing concern, and a point not to be overlooked, is the economic importance of many species of native wildlife that has increased in terms of their potential food value increasing the necessity for information about their parasites (Thompson, 1999).

To maintain the biodiversity and integrity of ecosystems, the nature and characteristics of parasite fauna must be known. Conservation programs now consider parasites might also be useful biomarkers, as they can be more sensitive to environmental stresses than their host populations (Landsberg *et al.*, 1998). Tasmanian farmers often observe native wildlife to monitor their property for disease (Driessen, personal communication). Populations of wild animals are regulated by many biotic and abiotic factors, which include parasites (Borgsteede, 1996; Tompkins and Begon, 1999). Within geographically isolated populations specific biotypes of agents adapt to individual species and distinct environments (Obendorf, 1995). Unfortunately when whole ecosystems are concentrated into remnant habitats, they can be subjected to a range of destabilising factors leading to the loss of biodiversity. The threat of disease outbreaks can increase as a result of crowding and more opportunities for transmission of pathogens or a lowered threshold of individuals to maintain the disease outbreak. The reduction in genetic diversity that often accompanies any population decline can further increase the risk of infectious diseases, a factor highlighted in species survival and recovery plans.

The investigations presented in this thesis reveal that *P. gunnii* is extremely susceptible to even low doses of *T. gondii* oocysts. The development of clinical disease is rapid, with high morbidity and mortality relating to a demonstrated deficiency in the cell mediated immunity. *P. gunnii* appears to be susceptible to other protozoan species including *Hepatozoon* and *Giardia*. The close association of *P. gunnii* with humans and the infringement of humans and cats into their habitat makes this marsupial vulnerable to trauma, predation, shelter and food deprivation, and exacerbation of parasitic and microbial diseases. Increased knowledge of the disease and fundamental cellular responses of *P. gunnii*, should lead to the development of strategies that control and reduce the infections of both wild and captive animals.

In summary, this study further clarifies diseases to which *P. gunnii* are susceptible; the relationship between humans, *P. gunnii* and parasite; and it also explores the possible zoonotic potential that exists within Tasmania. In the broader context, this study has shown the exciting possibilities that exist for basic microbiological, especially parasitological, research and this opens up great opportunities for molecular studies. The findings of previously unreported parasites in *P. gunnii* in this investigation support the recent reports and description of Flinders Island Spotted fever (Stewart, 1991) and new zoonotic helminth infections in humans in Tasmania – one by *Trichinella pseudospiralis* (Andrews *et al.*, 1994; Andrews *et al.*, 1995) and one for a new and completely unknown muspecoid nematode (Dennett *et al.*, 1998). As is the case of *Hepatozoon* and trypanosomes reported here, the epidemiology and extent of infection by these parasites remains to be eludicated. Additionally, with new human zoonotic infections being continually recognised, all studies on native animals which identify their microbial parasites and elucidate the natural history of these organisms, are to be encouraged.

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Chapter 11. Appendices

11.1 COMMON AND SCIENTIFIC NAMES OF VERTEBRATES SPECIES

MAMMALS
ORDER MONOTREMATA (MONOTREMES)
Tachyglossidae (Echidnas)
Short-beaked Echidna – *Tachyglossus aculeatus*

Ornithorhyndhidae (Platypus)
Platypus – *Ornithorynchus anatinus*

ORDER MARSUPIALIA (MARSUPIALS)
Didelphidae (American opossums)
Virginia opossum - *Didelphis virginiana*
South American gray short-tailed opossum - *Monodelphis domestica*

Dasyuridae (Dasyurids – Marsupial 'mice', 'cats' etc)
Brown antechinus (marsupial mouse) – *Antechinus stuartii*
Eastern Quoll - *Dasyurus viverrinus*
Spotted tail Quoll - *Dasyurus macalatus*
Tasmanian devil - *Sarcophilus harrisii*

Peramelidae (bandicoots)
Eastern barred bandicoot – *Perameles gunnii*
Southern brown bandicoot – *Isoodon obesulus*
Western barred bandicoot - *Perameles bougainville*
Desert bandicoot - *Perameles eremiana*
Long-nosed bandicoot - *Perameles nasuta*
Golden bandicoot - *Isoodon auratus*
Northern brown bandicoot - *Isoodon macrourus*
Pig-footed bandicoot - *Chaeropus ecaudatus*
Greater bilby - *Macrotis lagotis*
Lesser bilby – *Macrotis leucura*

Peroryctidae
Giant bandicoot - *Peroryctes broadbenti*
Raffray's bandicoot – *Peroryctes raffayanus*
Striped bandicoot – *Microperoryctes longicauda*
Mouse bandicoot – *Microperoryctes murina*
Papuan bandicoot – *Microperoryctes papuensis*
Large-toothed bandicoot – *Echymipera clara*
Kiriwina bandicoot – *Echymipera davidi* sp. nov.
Spiny bandicoot - *Echymipera kalubu*
Rufous Spiny bandicoot- *Echymipera rufescens*
Ceram Island bandicoot - *Rhynchomeles prattorum*

Phalangeridae (Brushtail possum, cuscuses)
Common brushtail possum – *Trichosurus vulpecula*
Potoroidae (Rat kangaroos)
Long-nosed potoroo -*Potorous tridactylus apicalis*
Phascolarctidae (Koala)
Koala – *Phascolarctos cinereus*
Vombatidae (Wombats)
Common wombat (Mitchell's wombat) – *Vombatus ursinus* (*Phascolomys mitchelli*)

Macropodidae (Kangaroos, wallabies)
Rottnest quokka – *Setonix brachyurus*
Tasmanian pademelon – *Thylogale billardierii*
Bennett's wallaby – *Macropus rufogriseus rufogriseus*
Tammar wallaby – *Macropus eugenii*
Forester kangaroo - *Macropus giganteus*
Eastern grey kangaroo – *Macropus giganteus*
Red kangaroo – *Macropus rufus*
Agile wallaby – *Macropus agilis*
Western grey kangaroo – *Macropus fuliginosus*
Black-faced kangaroo - *Macropus fuliginosus melanops*
Wallaroo - *Macropus robustus*

PLACENTAL MAMMALS

ORDER PRIMATES
Cebidae (New-world monkeys)
Squirrel monkey – *Saimiri sciureus*

ORDER CARNIVORA
Canidae (Dogs, foxes)
Dog – *Canis familiaris*
Dingo – *Canis familiaris dingo*
Fox - *Vules vulpes*

Felidae (Cats)
Cat – *Felis catus*

Procyonidae (Raccoons)
Racoon – *Procyon lotor*

ORDER PERISSODACTYLA
Equidae (Horses)
Horse – *Equus caballus*

ORDER LAGOMORPHA
Leporidae (Rabitts)
European rabbit – *Oryctolagus cuniculus*

ORDER RODENTIA
Muridae (mice, rats, voles, gerbils, hamsters etc.)
Mouse – *Mus musculus*
Mongolian gerbil - *Meriones unguiculatus*
Eastern Swamp rat – *Rattus lutreolus*
Ctenodactylidae (gundis)
Gundi/gondi - *Ctendactylus gundi*

ORDER CHIROPTERA
Australian fruit bats - *Pteropus poliocephalus*

MISCELLANEOUS
Reptiles
Tiger snake – *Notechis ater*
Lizards
Skinks

Birds
Ravens *Corvus tasmanicus*

11.2 PROTOZOAN AND HELMINTH ENDOPARASITES REPORTED FROM *PERAMELES GUNNII.*

PROTOZOA

APICOMPLEXA

Eimeria sp.

Toxoplasma gondii

Sarcocystis sp.

Klossiella sp.

CESTODA

CYCLOPHYLLIDEA

Hymenolepidae

Hymenolepis perimelidarum

NEMATODA

TRICHOSTRONGYLIDEA

Dromaeostrongylidae

Cercopithifilaria johnstoni

OXYUROIDEA

Labiobulura inglisi

PHYSALOPTEROIDEA

Phyaloptera sp

SEURATOIDEA

Seuratidae

Echinonema cinctum

Echinonema sp. n.

RHABDITOIDEA

Strongyloididae

Parastrongyloides australis

TRICHUROIDEA

Capillariidae

Capillaria sp. (undescribed)

Capillaria sp. (undescribed)

Capillaria sp. (undescribed)

METASTRONGYLOIDEA

Angiostrongylidae

Marsuptostrongylus bronchiali

FILAROIDEA

Onchocercidae

Peramelistrongylus skedastos

ACANTHOCEPHALA

Polymorphidae

Australidromis semoni

TREMATODA

DIGENEA

Cathaemasiidae

Mehlisia sp

(Obendorf and Munday, 1990; Beveridge and Spratt, 1996)

11.3 ARTHROPOD ECTOPARASTIES (MITES, TICKS AND FLEAS) REPORTED FROM *PERAMELES GUNNII.*

ARACHNIDA
ACARI: MESOSTIGMATA
Dermanyssidae
Haemolaelaps domrowi
Haemolaelaps marsupialis
Mesolaelaps antipodianus
Mesolaelaps australiensis

ACARI: IXODOIDEA
Ixodidae
Ixodes tasmani
Ixodes cornuatus

SIPHONAPTERA
CERATOPHYLLOIDEA
Pygiopsyllidae
Pygiopsylla hoplia
Pygiopsylea zethi

Stephanocircidae
Stephanocircus dasyuri

(Obendorf and Munday, 1990; Beveridge and Spratt, 1996)

Sex	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	RDW	HDW	PLT	MPW	PDW	PCT
female	3.40	6.68	132	0.35	51.7	19.8	383	16.6	23.0	107	2.7	69.3	0.000
female		8.02	145	0.42	52.1	18.1	347	15.2	27.3	320	5.4	68.1	0.002
female		8.19	149	0.42	51.3	18.1	354	14.5	27.3	213	5.2	73.7	0.001
female		7.51	144	0.39	51.7	19.1	370	15.1	23.3	107	3.1	82.2	0.001
female	2.35	6.21	115	0.32	52.0	18.5	356	14.3	25.4	75	3.2	84.9	
female		7.66	137	0.40	52.5	17.9	341	14.2	21.6	77	3.8	91.8	0.050
female		6.83	122	0.35	51.0	17.9	351	14.1	24.9	22	4.0	95.0	0.010
female	2.28	7.88	143	0.43	54.1	18.2	336	14.5	24.2	31	2.9	100.6	0.020
male	9.78	8.05	144	0.43	53.0	17.8	336	14.3	22.8	203	5.8	57.7	0.001
male	8.75	8.59	154	0.47	55.0	17.9	325	14.9	24.6	326	6.0	56.2	0.002
male	4.50	7.01	138	0.28	52.7	21.8	349	13.5	22.0	106	4.8	81.8	0.001
male		7.34	133	0.39	53.4	18.2	341	13.6	21.1	105	4.2	81.9	0.070
male	4.04	8.38	177	0.45	53.7	21.1	394	14.2	25.7	155	3.6	90.2	0.001
male	1.90	7.67	140	0.41	52.9	18.3	345	13.4	22.6	38	8.5		
male		9.62	157	0.48	49.4	16.3	330	13.6	23.5	125	5.7	71.6	0.001
male		6.59	117	0.34	51.7	17.8	343	15.1	23.5	65	6.2	71.2	0.050
male	8.65	9.16	167	0.50	54.9	18.2	332	13.9	22.3	53	5.0	83.7	0.000
male		7.28	133	0.37	50.3	18.2	363	13.3	25.3	28	3.6	89.4	0.020
male		8.85	149	0.44	50.2	16.8	335	13.9	23.9	56	3.1	94.8	0.000

Appendix 11.4a. Haematological data for male and female P. gunnii with no detectable blood-borne parasites.

Sex	WBC	RBC	HGB	HCT	MCV	MCH	MCHC	RDW	HDW	PLT	MPW	PDW	PCT
female		8.03	150	0.41	50.8	18.7	368	14.7	24.9	140	3.0	92.2	0.001
female	8.37	7.86	156	0.42	53.3	19.8	372	14.9	33.4	129	6.0	75.3	0.005
female		8.52	152	0.46	53.9	17.8	330	15.1	23.4	138	4.7	78.5	0.001
female	6.09	7.70	137	0.41	53.1	17.8	336	13.5	20.6	122	3.1	95.1	0.020
female		8.75	146	0.41	46.6	16.7	358	17.1	30.5	101	4.2	94.0	0.080
male	2.60	7.23	134	0.39	54.3	18.6	343	15.3	24.1	122	5.5	71.1	0.001
male		6.83	128	0.37	54.2	18.7	345	15.1	26.4	65	4.4	85.3	0.040
male	2.42	6.52	132	0.38	57.7	20.2	349	15.1	22.7	128	3.8	85.5	0.001
male	2.78	7.78	138	0.40	51.3	17.8	347	13.2	21.7	103	3.6	88.3	0.060
male		6.92	169	0.52	49.6	16.1	325	14.6	24.0	60	5.2	77.3	0.010
male	3.70	8.92	139	0.41	45.8	15.6	342	15.6	26.7	263	7.9	57.8	0.021

Appendix 11.4b. Haematological data of male and female *P. gunnii* with blood-borne parasites.

NOTICE
Modification or changes made in the recommended procedure may affect the stated or implied claims. Cellabs and its agents and distributors shall not be liable for damages under these circumstances.

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GIARDIA CELISA

INTENDED USE
The GIARDIA CELISA TEST is an *in vitro* enzyme immunoassay for the detection of *Giardia* antigens in faecal specimens.

INTRODUCTION
Giardia is a binucleated flagellate protozoan parasite which exists in two forms: a non-infectious, pear-shaped trophozoite, 9-30um infecting the small intestine and the highly infectious cyst form which is elliptical in shape and ranges in size from 6-10um (1). Survival outside its host varies greatly between the two forms; the trophozoite which is extremely labile, lasting only a matter of hours outside the body, while the cyst form may survive for several days in an external environment (1).
Giardia is a common cause of gastrointestinal disease world-wide (2,3) and of waterborne diarrhoea in the United States (1,3). The parasite is responsible for infections due to water contamination (4) and travellers have been found to contract giardiasis from endemic areas (5). Transmission also occurs by direct contact especially by asymptomatic carriers and by food contamination (6,7). High risk categories include young children, immunocompromised patients and those without previous exposure (8). More recently giardiasis has become a common sexually transmitted disease (9). *Giardia* has been found in all animal hosts studied (10) and animal faecal contamination especially of water is another route of transmission in humans (4). Giardiasis may therefore be regarded as a zoonosis (11).
Clinical manifestations of giardiasis range from asymptomatic carriage with the passing of cysts to chronic debilitating diarrhoea, weight loss and malabsorption (8,12,13).

PRINCIPLE OF THE TEST
Diagnostic methods of giardiasis detect *Giardia* by microscopy. Faecal specimens are examined by various techniques (14); these conventional methods are laborious and are dependent on the skill of the microscopist. The success rate of stool examination varies between 50-70% (15,16) and several specimens are usually necessary to establish diagnosis. Giardiasis may also be present in the absence of whole organisms and can be confused with other illnesses such as Crohn's disease and ulcerative colitis (17). When infection is present but parasites are not detected, sampling of duodenal fluid (14,16) detects trophozoites but this method is invasive and expensive. Antigen detection of faecal specimens by enzyme immunoassay provides an alternative method of establishing diagnosis and has been found to be sensitive and specific (18,19,20). Large numbers of specimens can be tested rapidly and objectively and the procedure is less labour intensive.
The GIARDIA CELISA detects Giardia antigens in a capture enzyme immunoassay. Antigens from faecal specimens are bound to microplates which have been coated with purified mouse monoclonal antibodies to *Giardia*. These antibodies react with *Giardia* antigens of molecular weights 30,000 and 66,000 which are the major antigens shared by both cysts and trophozoites (19). After a washing step, a second antibody, purified rabbit antibody to *Giardia* is added, followed by an anti-rabbit IgG conjugated to horseradish peroxidase. Enzyme activity is directly proportional to the concentration of *Giardia* antigen present in the sample and control. The enzyme activity is measured by adding the chromogenic substrate 3, 3', 5, 5'-tetramethyl benzidine (TMB) which produces a colour which can be detected visually or by measuring the absorbance in a spectrophotometer.

CONTENTS OF KIT
Each kit is sufficient for 96 tests.
1. Microwell strips coated with monoclonal antibodies to *Giardia* (packed in dessicant). 12, 1 x 8 breakable strips. Ready for use.
2. Rabbit anti-*Giardia* antibody. 200 X concentrate. 0.2mL.
3. Anti-rabbit IgG peroxidase conjugate. 100 X concentrate. 0.2mL.
4. Diluent buffer. Used to dilute antibody and peroxidase conjugate concentrates. Ready for use in 2 x 13mL.
5. Wash buffer. 10 X concentrate. 80mL.
6. Substrate Solution A. Ready for use. 4mL.
7. Substrate Solution B. Ready for use. 4mL.
8. Stopping solution. Ready for use. 8mL.

9. Positive control. Giardia antigen from culture. Ready to use. 2.7mL.
10. PBS tablets. For preparation of the buffer used to dilute the stool specimens.

NOTE: All reagents contain 0.01% thimerosal as a preservative.

MATERIALS REQUIRED BUT NOT PROVIDED:

1. Formaldehyde solution. (For preparing 10% Formalin to be used for stool specimen dilution)
2. Micropipettes with disposable tips.
3. 10mL centrifuge tubes for stool specimen preparation.
4. Wooden applicator sticks.
5. Distilled water.
6. Known *Giardia* negative stool specimen.
7. Spectrophotometer.

PRECAUTIONS

1. For *in-vitro* diagnostic use only.
2. Reagents should not be used after the expiry date shown on the label.
3. Do not mix components from different kits.
4. Thiomersal preservative added to all kit components is a poison. Exercise caution when handling these components.
5. Stool specimens, and all materials which have been in contact with them should be treated as potentially infectious and must be handled and disposed of accordingly.
6. The substrate should be protected from exposure to light.
7. The stopping solution is corrosive, avoid contact with skin, eyes and mucous membranes.

SPECIMEN PROCESSING

To be carried out the day before the assay. Stool specimens require diluting in 10% Formalin prior to testing.

- Preparation of 10% Formalin for diluting stool specimens.

Prepare PBS buffer by dissolving 1 PBS buffer tablet in 100mL of distilled water. Add 10mL of Formaldehyde to 90mL of the PBS buffer to make a 10% Formalin solution.

Stool specimens may be fresh, Formalin or SAF preserved and can be stored at 2-8°C with 0.1% sodium azide or at -20°C for up to a month. Diluted stool specimens may be stored at 2-8°C for up to 4 days.

Fresh Stool Specimens

Dilute the test and confirmed negative control stool specimens 1/10 in the 10% Formalin solution. This can be done by adding 1mL of stool to 9mL of 10% Formalin in a 10mL centrifuge tube. Mix thoroughly with an applicator stick to disperse the specimen. Seal the tubes and stand overnight at 2-8°C.

Preserved Stool Specimens

Mix specimen thoroughly and dilute 1/3 in 10% Formalin. This can be done by adding 1ml of stool to 2mls of 10% Formalin in a 10mL centrifuge tube. Mix thoroughly with an applicator stick to disperse the specimen. Seal the tubes and stand overnight at 2-8°C.

TEST PROCEDURE

- Positive and negative control (100µl) must be included in each assay.
- It is recommended that all samples be tested in duplicate.
- Calculate the amounts of antibody, conjugate, diluent buffer and substrate required and prepare just sufficient for each assay.
- Do not store or use diluted reagents from previous assays.
- All reagents and samples must be brought to room temperature before use.
- Skilful technique in pipetting, diluting of reagents and prevention of cross-contamination between wells is necessary to obtain reliable results.
- Thorough washing of the wells is required. They must be completely emptied and filled with the wash buffer to the top of the wells at each washing step.
- Once assay has started, do not allow microwells to dry.

1. Prepare the wash buffer. If crystals are present in the concentrate, warm to dissolve. Add the 80mL of concentrate to 720mL of distilled water and mix. Store at 2-8°C in wash bottle.
2. Remove the required number of microwell strips reseal the remainder in the foil bag and return to 2-8°C.
3. Fit the strips into the plate holder and press firmly. Number the strips on the plate and record the samples by reference to the number on the plate frame.
4. Carefully remove stool samples from the refrigerator. Dispense 100µL of the diluted stool supernatant to the corresponding microwell. Dispense 100µl of positive control and 100µl of diluent buffer (negative control) to designated control wells. The stool specimens may be centrifuged at 600G for 5 minutes if required.
5. Cover the plate with foil and incubate in a humidified chamber for 10 minutes, 37°C.
6. Flick out the test solution and rinse the wells four times with wash buffer. Fill the wells close to the rim with the wash buffer and empty completely. After the final wash, invert the wells onto a paper towel and tap vigorously to remove excess moisture. Alternatively, wash wells four times using an automated plate washer.
7. Dilute the anti-*Giardia* antibody 1/200 with the diluent buffer. (1mL of diluted reagent is sufficient for 2x8 wells). Dispense 50µL per well and incubate one hour, 37°C, as in Step 5.
8. Repeat Step 6.
9. Dilute the enzyme conjugate 1/100 with the diluent buffer. (1mL of diluted reagent is sufficient for 2x8 wells). Dispense 50µL per well and incubate for one hour,37°C, as in Step 5.
10. Repeat Step 6.
11. Combine together equal volumes of the TMB substrate Solution A and B and mix. (If the solution turns blue after mixing, discard and prepare fresh reagent). Dispense 50µL per well and incubate for 20 minutes at room temperature in the dark. Add 50µL of the stopping solution to each well.
12. Read the results visually or measure the absorbance in a spectrophotometer at dual wavelengths of 450nm and 620nm or on a single wavelength at 450nm.

INTERPRETATION OF RESULTS

1. **Visual**

If *Giardia* antigens are present in the stool specimens, the positive wells will show a yellow colour. Negative wells will have the same or less colour than the negative specimen.

2. **Spectrophotometer**

Note: If the absorbance was measured at 450nm only, the values will be slightly higher than those measured at the dual wavelengths of 450nm/620nm.

Calculation of Cut-off Value

Calculate the mean absorbance value for the negative controls and add 100% to obtain the cut-off value.

i) Specimens with absorbance values greater than the cut-off value are considered positive.
ii) Specimens with absorbance values less than or equal to the cut-off value are considered negative.

LIMITATIONS

1. Results of the assay should be interpreted together with the clinical symptoms of the patient.
2. If the reagents are not stored or used at the recommended dilutions, sensitivity and accuracy will be affected.
3. The assay should be performed on untreated, Formalin or SAF preserved stool specimens. Specimens pretreated with other fixatives have not been tested.
4. Proper preparation of the stool specimen is important in order to obtain optimal results.
5. The GIARDIA CELISA TEST should be used only for testing faecal specimens. Other types of specimens have not been tested.

CROSSREACTIVITY

Stool specimens found to have the following organisms were processed and tested by the GIARDIA CELISA and found to be negative:

Ancylostoma duodenale	*Ascaris lumbricoides* eggs	*Blastocystis hominis*
Chilomastix mesnili	*Cryptosporidium*	*Dientamoeba fragilis*
Endolimax nana	*Entamoeba coli*	*Entamoeba hartmanni*
Entamoeba histolytica	*Enterobius vermicularis* eggs	*Escherichia coli*
Hookworm eggs	*Hymenolepis nana*	*Iodoamoeba butschli*
Strongyloides stercoralis	*Trichuris trichiura*	

11.5B DETECTION OF *GIARDIA* SPP. USING MICROSCOPY AND ANTIGEN DETECTION USING THE COMMERCIALLY AVAILABLE *GIARDIA* CELISA KIT.

No. *P. gunnii*	Microscopy	*Giardia* CELISA	No. *P. gunnii*	Microscopy	*Giardia* CELISA
1	-	+	16	-	-
2	+	+	17	-	-
3	-	-	18	-	+
4	-	-	19	-	+
5	-	-	20	-	+
6	-	-	21	-	+
7	-	-	22	-	+
8	-	-	23	-	+
9	-	-	24	-	+
10	+	-	25	-	+
11	-	-	26	-	+
12	-	-	27	-	+
13	-	-	28	+	+
14	-	+	29	-	-
15	+	+	30	-	-
			31	+	-

BIOCENE

Ross River Virus Total Antibody

DEB- ELISA

Defined Epitope Blocking (DEB)-ELISA for the detection of Ross River Virus Specific Neutralising Antibody in Serum or Plasma

Catalogue Code : BIO-RRT
Version: RRVT002; September 99

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QUICK REFERENCE PROTOCOL FOR ROSS RIVER TOTAL ANTIBODY ELISA *	
PREPARATION OF REAGENTS	**TEST PROCEDURE**
A. Dilute Positive control and patient test serum : mix 500 µl dilution buffer + 50 µl Pos control and patient test serum, colour shifts from purple to blue	
	1. Dispense 50 µl per well of Neg calibrator (YELLOW) in triplicate, Pos calibrator (RED) in duplicate and diluted Pos control and patient test sera (BLUE) in singlicate. Incubate 60 min at 37°C in the resealable bag or in a 100% moist chamber.
	2. Dispense per well 50 µl conjugate, colour shifts in the well from blue to green. Incubate 60 minutes at 37°C in the resealable bag or in a 100% moist chamber.
B. Prepare wash buffer: mix per 8-well strip: 3 ml Wash Buffer (10x) + 27 ml distilled water	
C. Prepare TMB substrate. mix per 8-well strip: 0.9 ml TMB diluent + 100 µl TMB (10x).	
	3. Wash 5 times, Dispense per well 100 µl prepared TMB, Incubate in the dark 30 min at room temp.
	4. Add per well 100 µl stop solution, Read absorbance at 450 nm (optionally with 620 nm ref).

* Read the entire protocol before starting the assay.

INTENDED USE

The Biocene Ross River Virus Total Antibody DEB-ELISA is a Defined Epitope Blocking enzyme immunoassay for the detection of Ross River virus specific neutralising antibody in serum or plasma. It is an aid in the diagnosis of Ross River virus infections and for the detection of immune status. The assay must be performed strictly in accordance with the instructions set out in this protocol. No responsibility is taken for any loss or damage (except as required by statute) caused or arising from non-compliance with the instructions provided.

INTRODUCTION

Ross River virus belongs to the alphavirus group of arboviruses and causes a syndrome known as " epidemic polyarthritis ". Ross River virus is transmitted via mosquito bite, and is the most common and widespread arboviral disease in Australia. Disease occurs most commonly in adults 20 to 50 years of age. Symptoms of Ross River virus disease include arthritis, fatigue, rash, myalgia and mild fever. A significant proportion of patients will have symptoms for more than 12 months, emphasising the significant morbidity of the disease. Outbreaks of disease have been reported throughout Australia, Papua New Guinea and the Solomon Islands. A single major epidemic also occurred in Fiji and various Pacific Islands. Epidemic activity is associated with periods of heavy rainfall, flooding and tidal inundation of salt marshes and coastal wetlands. In northern, tropical Australia, transmission occurs year round. In southern Australia, transmission typically occurs in spring summer. Diagnosis is often made serologically.

PRINCIPLE OF THE ASSAY

Biocene Ross River Virus Total Antibody DEB-ELISA detects a single type specific neutralising epitope (2). The test measures total antibody and is species independent.
Purified, inactivated Ross River whole virions are coated onto the surface of microtitre wells. Test samples and controls diluted 1/11 are added to the plate and incubated for 1 hour. This is followed by the addition of Ross River specific HRP labelled, purified monoclonal antibody and further incubated for 1 hour. All unbound material is washed away. Colour is developed by addition of TMB substrate.The reaction is stopped and optical densities measured at 450 nm. If specific antibody is present in the sample it will bind to the antigen on the plate and effectively "block" the attachment of HRP labelled monoclonal antibody, thus reducing the amount of colour produced. Negative samples have high colour. Positive samples have low or no colour.

KIT CONTENTS

Cat.Code	Description	Quantity
RRTB	Microtitre plate (12 x 8 break apart wells) coated with inactivated, purified Ross River virus antigen	1 plate
RRPT	Positive Calibrator, Equine, ready to use, contains thiomersal as preservative	1.0 ml
RRNT	Negative Calibrator, Equine, ready to use, contains thiomersal as preservative	1.0 ml
RRPC	Positive Control, Equine, undiluted serum, contains Bronidox as preservative	0.5 ml
RRPO	HRP Labelled Ross River Neutralising Epitope Specific Monoclonal Antibody, ready to use	7.0 ml
TU	TMB Substrate, x10 Conc	1.5 ml
TDU	TMB Diluent, ready to use	15 ml
RRSD	Sample Diluent (PBS Tween, 2% FCS), ready to use, contains thiomersal as preservative	60 ml
RB	Wash Buffer, x10 Conc, contains thiomersal as preservative	60 ml
SS	Stop Solution, ready to use	15 ml
--	Resealable Bag	2 bags
--	Instruction Manual	1 pc

REAGENTS AND MATERIAL NEEDED BUT NOT PROVIDED

1. Pipettes to deliver volumes between 50 and 1000 µl.
2. Volumetric laboratory glassware
3. Distilled (or deionised) water .
4. 37°C Incubator .
5. Clean disposable plastic tubes for sample dilution (approx. 3 ml capacity)
6. Clean disposable plastic tubes for TMB (12 ml capacity)
7. Disposable absorbent towels .
8. Automatic plate washer (optional)
9. Microtitre Plate Reader, equipped for measurement at 450 nm (optional with 620 nm reference filter)
10. Vortex tube mixer.
11. Timer .

PRECAUTIONS

1. All reagents supplied are for *in vitro* use only.
2. Avoid contact of substrate and stop solution with skin and mucous membranes. If these reagents come into contact with skin , wash with abundant tap water.
3. Each well is ultimately used as an optical cuvette. Do not touch the undersurface of the strips; do not use damaged or soiled wells.
4. Use only components that are provided in this kit. Do not mix reagents of different batch numbers.
5. The reagents supplied should be used only as indicated in this instruction manual.
6. Do not test the sample diluent as a blank, it will give non-interpretable results.
7. Avoid the use of sodium azide as a preservative, as it can inhibit HRP.
8. The conjugate and substrate should be protected from prolonged exposure to light.

COLLECTION , HANDLING , STORAGE OF SAMPLES

Serum or plasma from any species can be used in this method. Samples may be stored at 4°C for up to 7 days. If longer storage is required , store at -20°C to -70°C. Avoid repeat freezing and thawing. Do not use grossly haemolysed or lipemic sera/plasma.
NOTE : Treat all human samples as potentially infectious.

RRV-TAB PROTOCOL

Wash Procedure

Efficient washing is a fundamental requirement of EIA's. It is essential that the wash procedure be carried out with care to obtain reproducible inter and intra assay results. Both manual and automatic washing can be performed.

Manual Wash

1. Empty the contents of each well by turning the strips in the holder upside down followed by a firm short vertical movement. Keep the strips tightened by pressing the sides of the strip holder.
2. Fill all wells ***to the rim*** (300-350 µl) with wash buffer. Take care to avoid carry-over.
3. Turn the strips upside down and empty the wells by a firm vertical movement.
4. Repeat this wash cycle (steps 2 and 3) **4 times.**
5. Place the inverted plate on absorbent paper towels and tap the plate firmly ***to remove residual washing solution in the wells.***
6. Do not allow any of the wells to dry out before the next reagent is dispensed. Therefore, proceed with the next step immediately.

Automatic Plate Wash

When using automatic plate wash equipment, check that all wells can be aspirated completely, that the wash buffer is accurately dispensed reaching the rim of each well during each wash cycle. The plate washer should be programmed to execute ***5 (five) cycles.*** After the last cycle, remove the wash buffer from the wells by tapping firmly the plate on absorbent towels.

Test Procedure

Allow all reagents to reach room temperature before use.

1. Dilute the positive control and samples 1:11 with sample diluent (50 µl test sample or control mixed with 500 µl sample diluent). *The sample diluent contains a sample delivery indicator: a colour change from purple to clear blue can be seen upon delivery of the sample and control.*

2. Dispense 50 µl of the negative calibrator in triplicate and the positive calibrator in duplicate. The Positive control and unknown samples are run in singlicate. Insert the plates into the resealable bag or cover to avoid evaporation.

3. Incubate for 1 hour at 37°C in a 100% moist environment.

4. Remove plate from the Incubator. **DO NOT WASH.** Add 50 µl of HRP conjugate to each well. Tap plate gently to mix. Insert the plate into the resealable bag or cover to avoid evaporation.

5. Incubate for 1 hour at 37°C in a 100% moist environment.

Dilution scheme for preparation of TMB		
Number of 8-well strips in use	TMB diluent	TMB (10x)
1	0.9 ml	100 µl
2	1.8 ml	200 µl
6	5.4 ml	600 µl
12	10.8 ml	1200 µl

6. Prepare the substrate solution by diluting TMB 1:10 in the TMB diluent. The TMB solution is crystalline below 15°C and should be handled at room temperature. Check that no crystals are present when pipetting. Mix per 8 well strip 0.9 ml TMB diluent with 100 µl TMB substrate (10x), see scheme. Stability of the substrate at working strength when kept in the dark and at room temperature is 4 hours.
Note: use only clean disposable containers.

7. Prepare the wash buffer: for each 8 well strip mix 3 ml of the wash buffer (10x) with 27 ml distilled water. The stability of the working solution is one month at room temperature or one year at +4°C.

11.7 HAEMATOLOGICAL AND BIOCHEMICAL VALUES OF *P. GUNNII* FROM VICTORIA

NORMAL VALUES HAEMATOLOGY AND BIOCHEMISTRY – EASTERN BARRED BANDICOOT (*Perameles gunnii*), of Victoria.					
PARAMETER	**Units**	**Mean**	**Std Dev**	**Min**	**Max**
Hb	g/dl	15.52	2.47	10.60	21.70
PCV	%	46.00	7.00	28.00	65.00
RCC	$x10^{12}$/L	8.79	1.40	6.14	12.70
MCV	Fl	51.27	8.24	4.90	61.00
MCH	Pg	17.38	2.61	0.17	21.00
MCHC	G/dl	33.72	1.85	30.00	39.00
TProt	%	53.40	11.77	8.00	88.00
Retic	$x10^9$/L	12.00	-	12.00	12.00
Total WCC	%	8.14	3.88	1.00	25.70
Neutr	%	31.36	21.58	3.00	88.00
Abs. Neutr	$x10^9$/L	2.72	2.26	0.25	12.06
Band Forms	%	2.00	2.83	0.00	4.00
Lymph	%	64.62	-	11.00	165.00
Abs. Lymph	$x10^9$/L	4.88	2.56	0.13	12.10
Mono	%	1.16	1.71	0.00	7.00
Abs. Mono	$x10^9$/L	0.21	0.20	0.01	0.75
Eosin	%	4.00	3.16	1.00	18.00
Abs. Eosin	$x10^9$/L	0.25	0.16	0.05	0.70
Baso	%	1.00	1.00	0.00	2.00
Abs. Baso	$x10^9$/L	0.02	0.03	0.00	0.04
Na	mmol/L	151.17	5.26	141.00	165.00
K	mmol/L	5.72	6.02	3.40	37.00
Cl	mmol/L	110.17	3.79	99.00	116.00
Bicarb	mmol/L	22.89	4.07	2.00	29.00
Urea	mmol/L	14.07	2.78	1.80	19.50
Creat	mmol/L	0.07	0.02	0.01	0.10
Tot Bili	µmol/L	5.94	4.53	1.00	30.00
Tot Prot	g/L	48.12	5.54	38.00	61.00
Albumin	g/L	31.15	3.81	23.00	37.00
Globulin	g/L	16.74	6.84	5.00	33.00
Alk Phos	IU/L	282.34	291.71	13.00	1729.00
Ast (Got)	IU/L	43.50	38.07	16.00	257.00
Alt	IU/L	74.30	28.70	42.00	175.00
Cpk	IU/L	445.88	649.52	38.00	323.00
Ldh	IU/L	1029.09	322.67	540.00	1973.00
Chol	mmol/L	3.16	1.24	1.40	5.80
Calcium	mmol/L	1.32	0.78	0.41	2.48
Phosphate	mmol/L	2.31	0.68	0.64	4.52

11.8 ASSESSMENT OF MITOGEN PROLIFERATION IN FREE-RANGING *P. GUNNII*

Stimulation Index (SI)											
Mitogen/ P. gunnii	**40 µg/mL**	**20**	**10**	**5**	**2.5**	**1.25**	**0.63**	**0.31**	**0.16**	**0.078**	**0.04**
CONA 15	1.39	10.61	90.09	508.80	681.91	285.51	74.75	18.13	1.96	0.37	
CONA 14	6.35	16.39	30.19	50.13	84.92	42.18	12.16	3.70	1.22	0.78	
CONA 37	8.43	46.91	86.58	168.99	152.85	83.46	29.11	11.71	0.89	0.91	0.49
CONA 87	0.78	1.01	1.59	366.27	180.41	188.58	51.39				
CONA 34	1.93	2.71	4.15	4.55	4.76	4.07	3.48	2.12	0.76	0.36	0.11
CONA 42	1.25	0.75	4.69	16.68	29.64	19.97	2.42	1.97	0.72	0.80	
CONA 21	22.32	33.98	45.50	39.23	32.33	17.72	5.86	2.16	0.52	0.80	
CONA 116	21.97	18.64	25.71	42.88	57.78	48.20	39.73	12.55	4.11	0.43	
CONA 10	54.86	98.71	89.22	7.41	8.21	1.76	1.06	0.90	0.51	0.28	
PHA 15	5.85	9.12	12.65	20.26	32.52	43.49	52.20	34.43	22.67	3.64	1.00
PHA 14	1.89	2.65	4.17	7.43	6.69	4.81	3.13	2.66	2.16		
PHA 37	3.53	6.88	9.04	15.74	38.87	36.36	30.84	29.24	20.57	13.12	9.24
PHA 87	0.99	1.31	1.92	1.23	1.48	0.72	0.87	0.88	0.52		
PHA 34	1.02	1.08	1.77	1.48	1.46	1.57	1.62	2.14	1.12	0.98	
PHA 42	6.95	11.84	23.34	40.86	82.84	86.91	85.06	44.74	23.11	2.27	
PHA 21	1.89	2.60	3.44	4.46	6.92	9.96	13.05	16.18	11.40	7.29	3.52
PHA 116	6.35	16.39	25.56	54.39	84.92	63.36	12.16	3.05	1.22	0.75	0.76
PHA 10	3.97	7.44	17.11	22.07	37.21	44.34	50.26	30.62	21.68	1.66	
PHA 91	7.06	61.28	143.78	152.88	83.48	46.92	29.12	11.71	0.80	0.71	0.41

Stimulation Index (SI)											
Mitogen/ *P. gunnii*	**100 µg/mL**	**50**	**25**	**12.5**	**6.25**	**3.13**	**1.56**	**0.78**	**0.39**	**0.19**	**0.1**
JAC 15	7.0	8.3	7.1	8.5	3.8	6.0	10.5	8.1	7.6	6.6	8.5
JAC 14	1.9	2.6	3.4	4.5	6.9	10.0	13.1	16.2	11.4	7.3	3.5
JAC 87	2.7	4.2	7.4	6.7	4.8	3.1	2.7	2.2	0.0		
JAC 34	16.7	47.4	88.9	53.1	31.7	31.5	20.0	8.2	4.0	2.1	1.7
JAC 21	1.1	2.4	3.1	2.9	1.9	1.4	0.5	0.0	0.0	0.0	
PWM 14	9.87	9.39	13.78	16.66	36.99	50.75	24.27	43.04	28.47	4.78	
PWM 37	1.47	2.30	7.18	4.39	0.98	0.37	0.51	0.15	0.18	4.78	
PWM 87	0.78	2.96	4.33	8.92	10.64	10.48	9.95	9.12	7.85	5.99	4.51
PWM 43	0.98	4.30	13.33	19.09	25.34	26.20	23.87	20.90	17.76	13.02	8.70
PWM 116	3.11	4.45	5.91	6.11	5.56	4.87	4.14	3.03	2.03	0.17	
PWM 21	3.18	2.41	7.65	12.48	14.92	17.64	13.21	14.03	9.87	7.15	4.66

11.9. ASSESSMENT OF MITOGEN PROLIFERATION IN CAPTIVE ANIMALS

Stimulation Index (SI)											
Mitogen/ P. gunnii	40 µg/mL	20	10	5	2.5	1.25	0.63	0.31	0.16	0.08	O.O4
CONA 1	0.79	2.40	6.72	141.32	230.46	77.20	21.27	5.56	0.73	1.74	2.15
CONA 2	1.25	0.70	0.75	4.69	16.68	29.64	19.97	2.42	0.94	1.51	1.00
CONA 3	2.85	58.76	97.57	32.92	9.02	2.35	0.32	0.72	0.90	0.41	0.35
CONA 4	5.04	36.57	40.68	17.23	3.95	2.46	1.15	1.08	0.81	1.36	0.37
CONA 5	18.60	35.75	33.17	14.91	9.17	1.17	1.98	3.78	0.91	0.83	0.76
CONA 7	10.63	27.55	86.68	89.76	63.28	53.11	30.80	2.01	0.34	0.56	0.62
CONA 8	6.35	16.39	30.19	65.44	84.92	42.04	1.22	3.70	1.22	0.75	0.76
PHA 1	2.21	6.18	18.32	45.84	1.60	0.24	0.18	0.14			
PHA 2	5.41	13.11	40.99	74.16	87.61	68.86	102.59	88.84	48.11	9.37	
PHA 3	5.28	12.22	15.83	9.16	12.40	3.96	1.51	0.82	0.59	0.54	
PHA 4	37.01	63.06	124.37	217.68	441.34	463.04	453.16	238.39	123.14	12.10	
PHA 6	2.27	2.04	2.20	6.55	19.45	25.21	58.09	8.33	2.04	0.15	
PHA 7	6.04	66.88	169.19	198.68	226.38	294.42	276.63	11.13	7.70	4.30	
PHA 8	1.11	2.63	5.47	4.46	63.85	27.84	19.21	4.01	0.63	0.57	

Stimulation Index (SI)											
Mitogen/ P. gunnii	100 µg/mL	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.19	0.1
PWM 1	9.05	14.15	31.43	27.40	25.93	32.12	45.76	42.85	43.73	27.33	
PWM 3	1.76	3.03	5.63	10.06	12.31	12.77	10.57	1.78	1.59	0.45	
PWM 6	3.19	2.41	7.65	12.49	14.94	17.36	13.22	14.05	9.88	7.16	
PWM 7	3.80	5.56	11.46	13.66	12.78	13.46	11.71	10.08	7.70	5.80	
LECTIN 1	4.99	19.09	21.36	30.96	17.85	32.99	29.62	5.80	12.61	0.35	0.30
LECTIN 2	1.39	5.57	5.23	3.41	2.95	2.81	2.48	1.87	1.57	0.86	0.81
LECTIN 4	3.54	2.40	10.36	17.96	10.76	10.81	15.06	14.59	6.66	3.33	
LECTIN 6	1.00	3.62	4.78	16.15	14.83	8.20	5.31	0.91	0.17	0.17	
LECTIN 7	1.71	1.57	1.44	0.91	1.54	0.97	1.03	0.65	0.72	1.35	

Stimulation Index (SI)											
Mitogen/ P. gunnii	100 µg/mL	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.19	0.1
JAC 1	3.67	4.31	14.49	15.42	25.34	32.00	20.10	22.49	10.01	5.02	
JAC 2	5.56	45.62	57.11	74.09	99.95	141.24	101.52	102.51	81.98	42.40	
JAC 3	1.85	1.63	2.18	1.60	1.87	2.09	2.38	1.83	1.04	0.65	
JAC 5	4.46	14.21	42.78	45.98	52.87	42.12	40.21	20.14	18.14	5.24	
JAC 7	4.46	25.00	51.22	81.12	89.78	120.03	98.05	90.10	59.52	42.40	
JAC 8	3.20	10.25	25.01	35.25	65.23	62.15	40.10	43.01	21.04	18.41	

11.10 ASSESSMENT OF MITOGEN PROLIFERATION IN CAPTIVE ANIMALS BEFORE EXPERIMENTAL *T. GONDII* INOCULATION AND POST INFECTION (PI).

Stimulation Index (SI)											
Mitogen/ P. gunnii	40 µg/mL	20	10	5	2.5	1.25	0.63	0.31	0.16	0.08	O.O4
CONA 01	0.79	2.40	6.72	141.32	230.46	77.20	21.27	5.56	0.73	1.74	2.15
CONA 01 PI	4.60	29.50	109.01	62.32	91.91	26.85	4.13	0.82	1.12	0.71	
CONA 02	1.25	0.70	0.75	4.69	16.68	29.64	19.97	2.42	0.94	1.51	1.00
CONA 03	2.85	58.76	97.57	32.92	9.02	2.35	0.32	0.72	0.90	0.41	0.35
CONA 03 PI	1.45	3.15	5.61	7.40	5.82	7.12	4.21	2.60	0.84	0.73	
CONA 04	5.04	36.57	40.68	17.23	3.95	2.46	1.15	1.08	0.81	1.36	0.37
CONA 05	18.60	35.75	33.17	14.91	9.17	1.17	1.98	3.78	0.91	0.83	0.76
CONA 07	3.43	53.76	107.09	192.67	174.15	14.46	16.03	2.06	1.95	1.11	0.54
CONA 07 PI	10.63	27.55	86.68	89.76	63.28	53.11	30.80	2.01	0.34	0.56	0.62
CONA 08	6.35	16.39	30.19	65.44	84.92	42.04	1.22	3.70	1.22	0.75	0.76
PHA 01	22.76	20.84	31.63	50.01	67.38	58.56	47.41	18.54	5.91	1.18	
PHA 01 PI	2.21	6.18	18.32	45.84	1.60	0.24	0.18	0.14			
PHA 02	5.41	13.11	40.99	74.16	87.61	68.86	102.59	88.84	48.11	9.37	
PHA 03	5.28	12.22	15.83	9.16	12.40	3.96	1.51	0.82	0.59	0.54	
PHA 03 PI	2.21	28.95	45.59	49.49	84.24	121.31	109.78	32.73	13.14	4.88	
PHA 04	37.01	63.06	124.37	217.68	441.34	463.04	453.16	238.39	123.14	12.10	
PHA 06	2.27	2.04	2.20	6.55	19.45	25.21	58.09	8.33	2.04	0.15	
PHA 07	6.04	66.88	169.19	198.68	226.38	294.42	276.63	11.13	7.70	4.30	
PHA 07 PI	9.25	7.68	22.42	11.28	8.85	16.24	4.53	2.42	3.67	2.44	
PHA 08	1.11	2.63	5.47	4.46	63.85	27.84	19.21	4.01	0.63	0.57	

Stimulation Index (SI)											
Mitogen/ P. gunnii	100 µg/mL	50	25	12.5	6.25	3.13	1.56	0.78	0.39	0.19	0.1
PWM 01	9.05	14.15	31.43	27.40	25.93	32.12	45.76	42.85	43.73	27.33	
PWM 01 PI	2.84	9.87	14.87	12.46	11.86	16.37	18.90	32.90	27.86	6.60	
PWM 03	1.76	3.03	5.63	10.06	12.31	12.77	10.57	1.78	1.59	0.45	
PWM 03 PI	3.40	7.38	9.52	8.90	8.54	4.36	2.30	0.12	0.11	0.15	
PWM 06	3.19	2.41	7.65	12.49	14.94	17.36	13.22	14.05	9.88	7.16	
PWM 07	3.80	5.56	11.46	13.66	12.78	13.46	11.71	10.08	7.70	5.80	
PWM 07 PI	5.48	4.60	4.99	8.39	7.29	7.70	6.35	9.04	8.12	5.48	
LECTIN 01	4.99	19.09	21.36	30.96	17.85	32.99	29.62	5.80	12.61	0.35	0.30
LECTIN 01 PI	13.24	3.76	7.64	10.44	17.74	17.99	13.78	10.66	1.39	1.38	
LECTIN 02	1.39	5.57	5.23	3.41	2.95	2.81	2.48	1.87	1.57	0.86	0.81
LECTIN 02 PI	1.58	5.54	2.92	3.16	3.04	2.51	7.68	6.91	1.35	0.97	1.25
LECTIN 04	3.54	2.40	10.36	17.96	10.76	10.81	15.06	14.59	6.66	3.33	
LECTIN 06	1.00	3.62	4.78	16.15	14.83	8.20	5.31	0.91	0.17	0.17	
LECTIN 07	1.71	1.57	1.44	0.91	1.54	0.97	1.03	0.65	0.72	1.35	

End piece. Tasmania, Australia.