Health and disease status of Australia's most critically endangered mammal the Gilbert's potoroo (*Potorous gilbertii*)

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A dissertation submitted to Murdoch University in fulfillment of the requirements of a Doctor of Philosophy.

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June 2008

"The aim of conservation medicine is ultimately to develop a solution-oriented, practice-based approach in addressing health problems derived from environmental change. This builds upon existing knowledge frameworks in wildlife health, public health, epidemiology, ecology, conservation biology, and veterinary science. By working at a larger scale of perspective, conservation medicine provides more specialized disciplines to interact in a more effective manner. In this way, conservation medicine employs concepts of 'consilience' by bringing together disciplines long separated by time and tradition" (Daszak 2004).

This thesis is presented as an applied research project embodied within the above framework. It represents a working example of Daszak's four guiding elements of conservation medicine constituting; interdisciplinary interaction, individual collaboration, institutional cooperation and innovative investigation (Daszak 2004).

I declare that this thesis is my own account of my research and contains as its main content, work which has not been previously submitted for a degree at any tertiary education institution.

Dr Rebecca Vaughan BSc BVMS

June 2008.

Acknowledgements:

This thesis is the result of extensive field work and laboratory investigation which would not have been achieved without the support and contributions of the people below.

Dr Tony Friend, Stephanie Hill and Tim Bunton from the Department of Environment and Conservation (DEC) in Albany who manage the ongoing recovery of the Gilbert's potoroo in the Two Peoples Bay Nature Reserve as well as Val Hack and other members of the Gilbert's potoroo action group (GPAG). I would like to acknowledge the time, ongoing dedication and effort of the Albany DEC staff and GPAG volunteers, for assistance in trapping, sample collection, processing, transport and friendship. It has been a pleasure to work with you all.

Dr Nicky Buller of the Department of Agriculture and Food WA (DAFWA) for extensive assistance, microbiology knowledge and funding support. Nicky's molecular knowledge and commitment to the underlying significance of the potential pathogenicity of isolated species was greatly appreciated.

My supervisors, Dr Kristin Warren, Dr Stan Fenwick and Dr Cree Monaghan. Thankyou so much for all your assistance and enthusiastic support throughout this project. In particular I would like to thank my principal supervisor Kris, who was always available to bounce ideas off, visited me in the field and provided me with help 'above and beyond' the call of duty. Kris highlighted the importance of this project from the early days and strove for funding and in doing so managed to secure the bulk of testing at no cost. Through her numerous contacts in conservation medicine and the DEC, Kris was able to ensure this project became a reality and evolved into what is undoubtedly one of the first comprehensive health and disease studies of an endangered marsupial species in Australia. In my 3rd year of my undergraduate veterinary degree Kris also provided me with the inspiration to pursue a wildlife and exotic animal veterinary career. This inspiration led me to then pursue a combined residency in zoo and wildlife medicine and a PhD, a decision I have never looked back upon.

My partner, Ben Higgins is to be thanked for endless proofreading, support and for understanding the importance of this project to me and conservation medicine.

Finally, I would like to thank my family; Dad, Mum, John, Lisa, Michael, Violet, Suzannah, Peter and Sara for always supporting me to achieve my goals, whatever they may be.

Abstract:

The Gilbert's potoroo *(Potorous gilbertii*) is a small marsupial endemic to the Two Peoples Bay Nature Reserve in the south-west of Western Australia. The Gilbert's potoroo is classified as Australia's most critically endangered mammal (IUCN 2006) with an estimated population of only 35 individuals. This thesis examines the health and disease status of the Gilbert's potoroo, presenting a strong case for the relatively new concept of disease as a potential threatening factor and modifier of population decline.

Specific diseases, including *Cryptococcus*, ectoparasitism, endoparasitism, haemoparasitism, *Toxoplasma* and a novel *Treponema* organism are extensively studied. An assessment of the clinical significance of these diseases is made, and management strategies are recommended to minimise the impact of these diseases on both the wild and captive population.

The novel *Treponema* organism which clinically presents with tenacious, green discharge and an associated balanoposthitis in males is molecularly characterized. Epidemiological studies show the effects of this agent on reproductive function and a penicillin-based treatment regime is trialled in the analogous long-nosed potoroo *(Potorous tridactylus)* with a recommendation to then trial this treatment regime in the critically endangered Gilbert's potoroo.

Standard haematological and urinalysis findings are tabulated to form reference ranges for this species. A treatment regime for *Cryptococcus* in the analogous long-nosed potoroo is reported and parasitological findings, including the identification of a novel tick species are discussed.

This thesis addresses key health issues, which have subsequently been incorporated into the Recovery Plan of the Gilbert's potoroo. A document encompassing multiple disciplines and expertise to support the recovery of this critically endangered marsupial in its current environment. In addition, this thesis outlines a recommended health monitoring and treatment protocol for future translocation procedures and provides a working example of the emerging importance of health monitoring in threatened species recovery programs.

Publications:

Scientific publications arising from this research:

Vaughan, R.J., Warren, K.S., Mills, J., Palmer, C., Fenwick, S., Monaghan, C.L., & Friend, J.A. 2008. Haematological and serum biochemical reference values and cohort analysis in the Gilbert's potoroo (*Potorous gilbertii*). *In press accepted for publication in the JZWM March 17 2008*.

Vaughan, R.J., Vitali, S.D., Eden, P.A., Payne, K.L., Warren, K.S., Forshaw, D., Friend, J.A., Horwitz, A.M., Main, C., & Krockenberger, M.B. 2007. Cryptococcosis in Gilbert's and long-nosed potoroo. *Journal of Zoo and Wildlife Medicine,* 38(4), 567-573.

Three manuscripts are currently in preparation.

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Glossary of abbreviations, acronyms and scientific names of marsupials.

AARL	Australian Rickettsial Reference Laboratory
Ab	antibody
Aepyprymnus rufescens	rufous-rat bettong or rufous-rat kangaroo
Ag	antigen
AGT	alanine-glyoxylate aminotransferase
AI	artificial insemination
ALP	alkaline phosphatase
ALT	alanine aminotransferase
ANOVA	analysis of variance
Antechinus flavipes	yellow-footed antechinus
Antechinus stuartii	brown antechinus
ARKS	animal record keeping system
AST	aspartate aminotransferase
Bettongia gaimardi	eastern bettong
Bettongia lesuer	burrowing bettong
Bettongia penicillata	brush-tailed bettong
b.i.d	twice daily
BFP	biological false positives
C	captive
°C	degrees celcius
CALAS	Cryptococcal Antigen Latex Agglutination System
CBC	complete blood count
CDC	Center for Disease Control and Prevention
CI	confidence interval
chip	microchip

CK	creatine kinase
cm	centimetre
CNS	central nervous system
CO_2	carbon dioxide
CODD	contagious ovine digital dermatitis
CSF	cerebrospinal fluid
DAFWA	Department of Agriculture and Food Western Australia
Dasyurus geoffroii	western quoll
Dasyurus hallucatus	northern quoll
DAT	Direct agglutination test
DD	digital dermatitis in cattle
DEC	Department of Environment and Conservation
Dendrogalus matschiei	Matschie's tree kangaroo
Dendrogalus ursinus	black-tree kangaroo
DFM	dark field microscopy
Didelphis marsupialis	American opposum
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EDTA	ethylenedinitrilotetraacetic acid
EENT	ear, eyes, nose and throat
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ET tube	endotracheal tube
F	female
g	gram
g	gravity force
G	gauge
GNR	Gram-negative rod

GP	Gilbert's potoroo
ha	hectare
H&E	Haematoxylin and Eosin
HIV	Human immunodeficiency virus
hpf	high power field
HR	heart rate
Hypsiprymnodon moschatus	musky-rat kangaroo
ID	identification
IFA	Immunofluorescence antibody
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IM	Intramuscular
Isoodon macrourus	short-nosed, northern-brown, or golden bandicoot
Isoodon obesulus	southern-brown bandicoot
IU	International Units
IUCN	World Conservation Union
IV	Intravenous
Juv	juvenile
km	kilometre
L	litre
LCAT	Latex cryptococcal antigen test
M	male
Macropus agilis	agile wallaby
Macropus antilopinus	antelopine kangaroo
Macropus bennetti	Bennett's wallaby
Macropus dorsalis	black-striped wallaby
Macropus eugenii	tammar wallaby

Macropus fulginosis	western-grey kangaroo or kangaroo-island kangaroo
Macropus giganteus	eastern-grey kangaroo
Macropus giganteus melanops	black-faced kangaroo
Macropus irma	black-gloved wallaby
Macrotis lagostis	bilby
Macropus parryi	Parry's wallaby
Macropus robustus	Euro, common or hill wallaroo
Macropus rufogriseus	red-necked wallaby
Macropus rufus	red kangaroo
MAT	Modified agglutination test
MBD	methylene blue dye binding
mg	milligram
$MgCl_2$	Magnesium chloride
mL	milliliter
Myrmecobius fasciatus	numbat
n	number
NAD	no abnormalities detected
neg	negative
NSAID	non-steroidal anti-inflammatory drug
OIE	World Organization for Animal Health
p	p-value or probability value
PHA	Mitogen driven proliferation assays
pos	positive
py	pouch young
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
Perameles bougainville	western-barred bandicoot
Perameles gunnii	eastern-barred bandicoot

Petaurus breviceps	sugar glider
Petrogale penicillata	Victorian brush-tailed rock wallaby
PHS	Potoroo hyperoxaluria Syndrome
PO	oral route
Pseudocheirus peregrinus	ringtail possum
Onychogalea fraenata	bridled nail-tail wallaby
Onychogalea unguifera	northern bridled nail-tail wallaby
OR	odds ratio
Ornithorhynchus anatinus	platypus
Permales nasuta	long-nosed bandicoot
Petrogale brachyotis	short-eared rock wallaby
Petrogale concinna	pygmy rock wallaby
Petrogale herberti	Herbert's rock wallaby
Petrogale inornata	unadorned rock wallaby
Petrogale penicillata	brush-tailed rock wallaby
Petrogale persephone	Proserpine rock-wallaby
Phascolarctos cinereus	koala
Potorous gilbertii	Gilbert's potoroo
Potorous longipes	long-footed potoroo
Potorous tridactylus	long-nosed potoroo
q24hr	every 24 hours
RBC	red blood cell
RNA	ribonucleic acid
rRNA	ribosomal RNA
rpm	resolutions per minute
RPR	Rapid plasmid reagin
RR	respiratory rate
Schoinobates volans	marsupial glider

Setonix brachyurus	quokka
s.i.d	once daily
SC	Subcutaneous
Sminthopsis murina	slender-tailed dunnart
sp.	species (singular)
spp.	species (plural)
Tachyglossus aculeatus	short-beaked echidna
Tarsipes rostratus	honey possum
TBE	Tris/Borate/EDTA
Thylogale billardierii	Tasmanian pademelon or rufous-bellied pademelon
Thylogale stigmatica	red-legged pademelon
t.i.d	three times daily
TPPA	*Treponema pallidum* particle agglutination
Trichosurus caninus	mountain brushtail possum
Trichosurus vulpecula	brushtail possum
µg	microgram
US	United States
UTI	urinary tract infection
UV	ultra-violet light
V	volts
VDRL	Venereal Disease Research laboratory
VGI	*Cryptococcus gattii* (molecular type I)
VGII	*Cryptococcus gattii* (molecular type II)
Vombatus ursinus	wombat
W	wild
WA	Western Australia
Wallabia bicolor	swamp wallaby or black wallaby
Wallabia eugenii	tammar wallaby

WBC	white blood cell

CHAPTER 1

Introduction

1.1 GENERAL OVERVIEW

Since 1500 AD it is estimated that 698 animal species have become extinct (IUCN 2006). Around the world, wildlife populations continue to shrink. The figures delineating threatened species status vary considerably pending the criteria used to define their status. However, the World Conservation Union (IUCN) estimates that at least 11,000 known animal and plant species are threatened with extinction, which is between 1,000 and 10,000 times the natural extinction rate (Steiner 2003). Natural selection and extinction of species are considered a natural part of the evolutionary process. However, in recent times a human induced shift in dynamics seems to be driving species decline through factors such as overexploitation and direct environmental change, for example habitat loss (Rosser and Mainka 2002).

Australia has had a disproportionately high number of mammalian extinctions compared to other countries (Caughley and Gunn 1996). These animals have shown similar biological and ecological characteristics, being medium-sized mammals (35 to 5500g) that were generally herbivorous and inhabitants of semi arid and arid regions. These medium-sized mammalian extinctions are often attributed to habitat loss, changing land-use and fire regimes, and adverse impacts of introduced species through predation and competition. Globally threatening agents that have been directly linked to, or postulated as the cause of population decline, driving species to extinction, include habitat modification, introduced animals, over exploitation, climate change, environmental contaminants, inbreeding depression and disease.

1.2 CAUSES OF POPULATION DECLINE IN WILDLIFE

1.2.1 Habitat modification

1.2.1.1 Habitat loss

In Australia land clearance, mainly for agriculture is the single most important cause of environmental degradation, and loss and depletion of species and ecological communities (Clark *et al*. 1990). Worldwide, habitat loss accounts for the population decline of almost 80% of the mammals and about 60% of the birds listed by the IUCN as threatened (Groombridge 1992). Extensive habitat loss is a feature of all biodiversity 'hot spots' (Pimm and Raven 2000). These 25 'hot spots' of biodiversity contain half the world's vascular plants species and one-third of the world's terrestrial vertebrate species. None of these hotspots have more than one third of their original habitat remaining and habitat loss continues to be a significant problem in many of these regions. As such the wildlife populations in these areas are threatened with decline and extinction (Brooks *et al.* 2002.)

1.2.1.2 Fragmentation

Fragmentation is the process of permanent land clearance or habitat modification which leaves behind small, isolated remnants of native vegetation amongst agricultural, urban or otherwise disturbed land (Burgman and Lindenmayer 1998). This ultimately results in the formation of 'island ecosystems' isolating species geographically and confining them to smaller areas (Deem *et al.* 2001). Isolation can promote a long term loss in genetic diversity (Frankel and Soule 1981) and, associated with this, a potential loss of evolutionary adaptation (Lyles and Doson 1993).

1.2.1.3 Changing land use patterns

Alterations of land use patterns can lead to significant habitat modification (Warren 2007). Land clearance for agriculture, forestry plantations and improved pasture is considered to be

the leading cause of global environmental degradation, leading to population decline. One of the consequences of land clearance is that remnant vegetation is subjected to altered physical and biological processes (Burgman and Lindenmayer 1998). In Australia, the phenomenon of 'rural dieback' has caused premature decline and death of trees in a wide range of forest and woodland types. A single, universal cause of dieback has not been identified (Landsberg and Wylie 1983) although factors resulting in rural dieback are suspected to vary between regions (Landsberg and Wylie 1991). In the south-west of Western Australia, dieback is presumed to be caused by an introduced soil-borne fungus, *(Phytophthora cinnamoni)* (Orr *et al.* 1995) and is spread through the movement of fungal spores in water and moist soil. The disease is thought to be perpetuated by a wet climate and the movement of vehicles and humans and animals traversing from infected to uninfected areas. Numerous other changes in land use patterns such as mining and urbanization can also contribute to species decline.

1.2.1.4 Land degradation

Land degradation is effectively any process whereby land deteriorates due to the actions of the natural agents of wind, water, gravity and temperature, and is characterised by a reduction in soil depth or water quality (Blyth and Kirby 1984). Worldwide, land degradation has been estimated to affect 20% of the earth's vegetated land (World Resources Institute 1992). The geographical processes of land degradation, although natural in origin are often accelerated by human induced change. For example, in Australia land degradation usually occurs in conjunction with agricultural development. Examples of land degradation include wind and water erosion, dry land salinity, irrigation salinity and water logging, soil structure decline, vegetation degradation, mass movement of soil, chemical contamination of soil and water and soil acidification (Mercer 1995). Land degradation is considered to be a major threatening factor for fragmented patches of remnant habitat and conservation of biodiversity in these regions.

1.2.2 Introduced animals

Historically, introduced animals have been implicated in an estimated 40% of extinctions globally (Caughley and Gunn 1996). In Australia the introduction of foxes, rabbits, goats, feral dogs and cats, and many avian and aquatic species has drastically affected both directly and indirectly the extinction of many native Australian mammalian species. Habitat destruction caused by these introduced species is a major threatening agent. However, populations already compromised in numbers can also be driven to extinction through predation, competition, or other forms of disturbance from these introduced species (Caughley and Gunn 1996).

1.2.3 Over-exploitation

1.2.3.1 Trade

The livelihood of a large proportion of people especially in third world countries is dependent on wildlife species for trade and subsistence use. There is local, national and international demand for wildlife species and their products for food, medicine, ornaments, furnishings, clothing, pets, manufacturing and construction (Broad *et al*. 2003). Trade challenges biological diversity through over-exploitation, through the catching of non-target species and through the introduction of harmful, exotic species (Broad *et al*. 2003). It is difficult to quantify the economic activity generated through wildlife trade given its lack of regulation and documentation. However, estimates of the number of people dependent on wildlife trade for income range from 200 million in the Asia-Pacific region alone to one billion worldwide (van Rijsoort 2000).

1.2.3.2 Subsistence use

The richest areas of biodiversity are those with the highest number of threatened species, and these areas also have a high number of malnourished people of low economic standing (Mainka 2002). As such complications arise in achieving the balance needed to ensure the

survival of species and the livelihoods of those dependent on these species. Mace and Balmford (2000) recorded 1102 major threats to 600 species of mammals, including non-threatened species. Over-exploitation, including use of species for food and body parts, ranked as the second most common threat following habitat loss, and most commonly occurred for human consumptive use rather than ornamentation.

1.2.4 Climate change

Scientists and policy makers agree that the world's climate is changing at an unprecedented rate and that global warming is occurring (Burgman and Lindenmayer 1998, Hannah *et al*. 2002). Global warming is a result of human activities, and on a *per capita* basis Australia is responsible for releasing among the greatest volumes of greenhouse gases, with a large proportion of these emissions being associated with agriculture (Burgman and Lindenmayer 1998). The effects of global warming are predicted to have major implications on biodiversity. Changing environmental processes such as the availability of water, changing rainfall patterns, temperature ranges and extreme climatic events (e.g. the El Nino Southern Oscillation) may result in a shift in the distribution of many species (Hannah *et al.* 2002). It is possible that many ecosystems presently suitable for particular wildlife species may be unable to support these species in the future (Hannah *et al.* 2002). Global climate change has occurred over the past two million years, however, changes in the near future are expected to occur far more rapidly than those seen in the past (Burgman and Lindenmayer 1998). It is possible that the rapidity of these changes will exceed the ability of some species to adapt. Therefore dispersal abilities, persistence of established populations and competitive abilities will determine the future distribution and abundance of many species (Hannah *et. al* 2002).

1.2.5 Environmental contaminants

Environmental contaminants are often suspected, and in some cases have been proven to cause population decline (Caughley and Gunn 1996). There is increasing concern over the effects of contaminants causing endocrine disruption on the reproductive success and survival of wildlife populations (Smolen and Colborn 1997). In Australia, the increased use of pesticides, fungicides and herbicides has the potential to cause far reaching adverse environmental effects. Many organic chemicals used are fat soluble, and as such accumulate in the fatty tissues of animals. If lower order prey are ingested by higher order carnivores the chemical is concentrated, increasing the potential for harm (Burgman and Lindenmayer 1998). Nutrient rich sediments, agricultural fertilizers, domestic sewage and industrial effluent can lead to eutrophication, algal blooms and resultant deoxygenation of inland waters leading to conditions that freshwater species are unable to tolerate (Brown *et al*. 1990). Oil spills and exploration can also have a direct impact on marine ecosystems. Apart from causing adverse effects on plants, animals and coastal habitat, oil contains hydrocarbons that may have lethal, sub-lethal and carcinogenic effects on marine mammals, birds, fish and invertebrates (Amos *et al*. 1993).

1.2.6 Inbreeding depression

Inbreeding depression resulting in reduced fitness has been postulated as a factor driving population decline (Caughley and Gunn 1996). Diminished genetic variation between populations reduces the opportunity for adaptive responses to variable local conditions, and may result in reduced reproduction or survival (Madsen *et al*. 1996). Although well documented in captive animal studies, the consequences of inbreeding in small populations in the wild remains unknown (Caughley and Gunn 1996). This is because of the inherent difficulties in attributing inbreeding to reduced fitness and assigning its significance to population decline (Caughley and Gunn 1996). However, in Australia Montgomery *et al.*

(2001) has documented a loss of reproductive capacity associated with inbreeding depression in a specific population of wild male koalas.

1.2.7 Disease

Disease leading to alterations in health status can place a significant challenge on individual species and populations, especially in those already compromised by other threatening agents. Disease has been shown to be the cause of significant population decline in a number of endangered species including the black-footed ferret (*Mustela nigripes),* due to canine distemper virus, the heath hen (*Tymphanuchas cupido cupido),* due to blackhead and the Indian wild ass (*Equus hemionus khur)*, due to surra and African horse sickness (Caughley and Gunn 1996). The importance of disease will be discussed later in this introductory chapter.

1.3 CONSERVATION MEDICINE

Understanding the nature and dynamics of the issues driving population decline requires broad expertise. A fundamental interdisciplinary approach is required linking scientific approaches from both the health and, the environmental conservation fields (Ostfeld, Meffe and Pearl 2002). It is the application at the interface of these separate disciplines that embodies the newly emerging field of conservation medicine.

Conservation medicine focuses on the multiple interactions between pathogens and disease, and between species and ecosystems. It represents an approach that is multi-disciplinary in examining the inter-connected nature of human, animal and ecosystem health (Meffe 1999, Pokras *et al.* 1999, Norris 2001, Tabor *et al.* 2001).

This new discipline was established in response to increasing concerns about the adverse effects of anthropogenic environmental change on human and animal health and owing to increasing evidence that disease played an important role in driving population decline and extinction of wildlife species (Meffe 1999, Deem *et al.* 2000, Weinhold 2003). The principal goals of conservation medicine are to develop scientific understanding of the relationship between the environmental crisis and human and animal health, and to develop solutions to problems at the interface between environmental and health sciences (Ostfeld, Meffe and Pearl 2002). Through its very nature, conservation medicine has developed in response to a web of problems that require urgent action and that generally are beyond the scope of a single health or environmental discipline.

Conservation medicine has been termed a 'crisis discipline', as action is often taken without complete knowledge. Many conservative scientists berate actions taken when the outcomes are uncertain. In many cases, and in particular with regard to endangered species, the cause for the original population decline is unknown or has only been postulated. The case of the Aleutian Canada goose, (*Branta canadensis leucopareia)* highlights the potential resource and monetary losses that can occur when conservation projects are based on preconceived ideas or assumptions of threatening processes (Caughley and Gunn 1996). Arctic foxes (*Alopex lagopus)* were thought to be responsible for the decline of the Aleutian goose, as such foxes were eradicated from their habitat on four islands in the Bering Sea. Yet the decline continued, and hunting pressure at other locations was later discovered to be the threatening process (Caughley and Gunn 1996).

However, as Peterson (1991) states, 'doing something that might be beneficial is better than doing nothing when the facts are unclear and the problem appears to be critical.' Other conservation biologists take the minimalist approach insisting that human intervention should not be provided in any way for wild animals as such action would interfere with evolutionary processes (Deem *et al.* 2001). Yet as Redford and Richter (1999) argue, there

are probably few species that have not been influenced by some form of 'human intervention.'

With regards to threatened wildlife management issues it is difficult to broadly dictate when, and when not, intervention is in the best interests of the target species without first determining the most appropriate recovery options on an individual species basis. This in itself necessitates broad ranging expertise of the target species and its current environment to enable an effective management protocol to be formulated. This approach is in line with current medical and veterinary clinical case management as it involves the creation of a list of differential diagnoses of threatening processes which are then individually assessed before an appropriate treatment regime or management plan can be executed (Caughley and Gunn 1996). The threatened species management plan should frame the management of the species within an ecological context and draw together the expertise of veterinarians, physicians and conservation biologists, and may involve further collaboration with social scientists, political scientists, economists, educators, policy makers and conservation managers (Meffe 1999, Osofsky 2000) to reach a mutually suitable management plan.

1.4 THE IMPORTANCE OF DISEASE IN POPULATIONS

Disease has been defined by Spalding and Forrester (1993), to constitute, 'any impairment that interferes with or modifies the performance of normal functions, including responses to environmental factors, such as nutrition, toxicants, and climate; infectious agents, inherent or congenital defects, or a combination of these factors.'

Disease, apart from causing death, can also increase the susceptibility to predation, lower the reproductive capacity, increase susceptibility to other disease or lead to a combination of these outcomes (Spielman 2001).

In the 1930's, Aldo Leopard, a leading figure in wildlife management in North America stated that the 'role of disease in wildlife conservation has probably been radically underestimated.' (Leopold cited in Spalding and Forrester 1993). Many ecologists and wildlife biologists continue to overlook the possibility of disease as a determinant of population dynamics. A retrospective study of terrestrial vertebrate translocations within conservation projects in the United States, Australia, New Zealand and Canada between 1973 and 1986 revealed that of the 700 translocations conducted per year, 24% of projects did not incorporate disease screening and less than 25% investigated the causes of mortality of translocated animals (Daszak *et al.* 2000).

However, a review of the literature reveals that in the 1970's in the United States (US) a number of surveys incorporating epidemiological approaches to free-ranging wildlife populations were undertaken (Deem, Karesh and Weisman 2001), and during the 1980's, following the passage of the US Endangered Species Act (Spalding and Forrester 1993), further funding became available for threatened species management sparking increased interest in the area.

Prior to this, little attention had been placed upon the way disease affects the distribution and abundance of populations. Reasons proposed for this lack of disease monitoring included the inaccessibility of many wildlife populations, necessitating extensive logistical and monetary support, and the argument that sick animals would be removed by scavengers and predators. This selective removal was thought to mask the effects of a disease and to lead to only severe epizootics being noticed (McCallum and Dobson 1995). Furthermore, by the time a population reached serious decline other interacting factors and chance environmental events often came into play (Spalding and Forrester 1993).

Prior to the 1970's diseases of wildlife were addressed only in relation to the health of domestic species (ruminants, pigs, chickens) coming into contact with wildlife, or if they

were economically important (game species, such as deer and ducks) (Friend and Trainer 1974, Pastoret *et al.* 1988, Plowright 1988). Zoonotic diseases such as rabies, brucellosis and tuberculosis were the other concern prompting further investigation of wildlife disease and reservoirs (Friend and Trainer 1974).

Therefore, extinction of a wildlife species due to infectious disease has not commonly been reported. Only one such report exists, that belonging to the Partula tree snail (*Partula turgida),* (Daszak and Cunningham 1999). Pathological investigation of the last individuals to die revealed a disseminated microsporidian infection (*Steinhausia* spp.) to be the cause of disease. However, numerous reports exist that exemplify secondary effects leading to extinction of species further up the food chain. For example the extinction of the eelgrass limpet (*Lottia alveus),* was intrinsically related to an outbreak of eelgrass wasting disease. This disease caused a loss of 90% of eelgrass cover on the United States (US) seaboard, with the host-specific eelgrass limpet becoming extinct shortly thereafter (Daszak and Cunningham 1999).

Amphibian chytridiomycosis is perhaps the most recent, commonly cited cause of mass mortality and population decline in amphibians. It has spread throughout Australia, North America, South America, Europe and New Zealand, and has been linked to local and possible species extinction, (Daszak *et al*. 1999, Bosch *et al.* 2000, Waldman *et al.* 2000). One affected species, the sharp snouted day frog (*Taudactylus acutirostris*), is now almost certainly extinct (Schloegel *et al*. 2006). The fungal infection caused by *Batrachochytrium dendrobatidis,* leads to a hyperkeratotic skin reaction which has been proposed to impede supplementary respiration, or osmoregulation through the skin, a vital requirement for amphibian physiology. Toxins may also be released during infection (Daszak *et al.* 2001).

Declining amphibian populations were previously hypothesized to be associated with habitat loss, increased ultraviolet-B-irradiation, global climate change, chemical pollution,

introduced predators, acidification, and unidentified environmental stressors causing immunosuppression (Daszak *et al.* 2001). However, it was only when veterinary examination of carcasses in Australia and later Panama was undertaken that a novel non-hyphal fungus *Chytridiomycota* was identified (Berger *et al*. 1998). Koch's postulates were later fulfilled demonstrating its pathogenicity in both Australian (Berger *et al.* 1998) and North American frogs (Longcore *et al.* 1999, Pessier *et al.* 1999).

Chytridiomycosis is perhaps one of the ultimate examples of an emerging infectious wildlife disease affecting multiple species on a panzootic scale. It is a disease which has become of paramount importance in terms of quarantine, husbandry, health, breeding and management procedures of both captive and wild amphibian populations.

As demonstrated, disease has the potential to cause significant morbidity or mortality in wildlife. However, it is also important to realize that 'less visible' diseases, capable of causing low-level mortality or sub-lethal effects on reproduction can also have significant demographic effects (Spaulding and Forrester 1993). These 'less visible' diseases can become catastrophically important to endangered species where population levels are critically low (Harwood and Hall 1990). These populations can also lose genetic variability as a result of random stochastic processes (i.e. genetic drift) or inbreeding and this may in turn increase susceptibility to disease (Hutchins *et al*. 1991).

In Western Australia the management of endangered species is commonly addressed through a Recovery Plan executed by the Department of Environment and Conservation (DEC). A Recovery Plan aims to delineate, justify, and schedule management options necessary to support the recovery of threatened species and ecological communities.

Recently, Recovery Plans have given increased consideration to the implications of disease on population dynamics and the possibility of it acting as an agent driving population

decline. This should in turn prompt provisions and funding for disease monitoring, and ensure that disease surveillance plays a pivotal role in reintroduction and translocation management. Wildlife management strategies aimed at increasing population numbers such as captive breeding colonies and translocation can also, if appropriate precautions are not taken, facilitate the spread of disease. As such, studies are needed to first assess the health and disease risks to the target species in their current environment, and secondly, to assess the health and disease risks to both the target species and the wild co-habitants in the release environment.

The Recovery Plan of the Gilbert's potoroo *(Potorous gilbertii)* Australia's most critically endangered mammal is a working example of the emerging importance of health monitoring in threatened species recovery programs. The Recovery Plan of the Gilbert's potoroo encompasses multiple disciplines and expertise to support the recovery of this critically endangered marsupial in its current environment, with subsequent plans for future reintroduction and widespread translocation to assure its long term survival.

1.5 THE GILBERT'S POTOROO IN THE CONTEXT OF CONSERVATION MEDICINE

The Gilbert's potoroo is a small potoroid endemic to the Two Peoples Bay Nature Reserve near Albany in the south-west of Western Australia. According to IUCN criteria (IUCN 2006) the Gilbert's potoroo is classified as Critically Endangered (Courtenay and Friend 2004). The Gilbert's potoroo belongs to the family Potoroidae. Genetic studies have found that this extant species is distinct from the two species of potoroo found in eastern Australia, the long-nosed potoroo (*Potorous tridactylus)* and the long-footed potoroo *(Potorous longpipes)* (Sinclair and Westerman 1997). However Sinclair *et al.* (2002) has also reported a genetic bottleneck in the Gilbert's potoroo, consistent with a demographic decline, highlighting the concern for the long-term survival of this species.

The Gilbert's potoroo was first collected by John Gilbert in 1840 at King George's Sound near Albany in Western Australia. It appears from early records that the Gilbert's potoroo has always been restricted to the high rainfall areas of the south-west of Western Australia, and was locally abundant in the areas in which it was found (Courtenay and Friend 2004).

The Gilbert's potoroo was presumed extinct, as there had been no sightings reported after 1870. However, in 1994 a small population was rediscovered at Mount Gardner, in the Two Peoples Bay Nature Reserve. This remnant population is restricted to a 1000 ha region of heath-land within the reserve. An interim recovery plan was prepared for the species immediately after its rediscovery (Start and Burbidge 1995). This plan recommended that a captive breeding program should be established as part of a comprehensive recovery program to insure against the catastrophic loss of the wild population (e.g. through wildfire) and to breed individuals for translocation to establish new populations. Eight animals (five adults, comprising one male and four females, a juvenile and two pouch young) were removed from the wild to establish the foundation group for the captive breeding program.

This breeding program has resulted in eight captive-bred young being raised over a ten-year period (Friend 2005). The reason behind this poor reproductive success is presently unknown but possibilities such as husbandry, diet, behavioural incompatibility, genetic or disease factors should be considered. The current captive population comprises one male and one female (T. Friend, personal communication, February 12, 2008).

Although a healthy rate of reproduction is evident in the wild population, researchers have been unable to re-trap a significant proportion of previously recorded pouch young. From 2002-2004, 38 pouch young were recorded, however only seven are known to have reached adulthood (Courtenay and Friend 2004). Potential reasons for this high rate of juvenile loss, include a limited carrying capacity for the Mount Gardner area, loss during pouch life, a high rate of predation during the dispersal phase, and dispersing young leaving the Mount

Gardner region and being lost to the population (Courtenay and Friend 2004). Research conducted on Gilbert's potoroos prior to this health study focused primarily on ecology, population, biology, reproduction and nutrition.

1.6 BACKGROUND INFORMATION REGARDING THE GILBERT'S POTOROO

1.6.1 Biology

The Gilbert's potoroo is a nocturnal macropodid marsupial that belongs to the family Potoroidae (Tyndale-Biscoe and Renfree 1987). There are approximately 50 extant species of Macropodids found in Australia. These are divided into two families, the Potoroidae comprising the smaller macropods including the potoroos, bettongs and musky rat kangaroos; and the Macropodidae including the larger macropods such as red and grey kangaroos, quokka and wallabies (Blyde 1999).

The body weight range of adult Gilbert's potoroos ranges from 900 to 1200g (Courtney and Friend 2004). Their body is densely furred, except for the tail, which aids in balance for hopping. Like other macropods, they possess short forelimbs and large hind limbs. The forelimbs have long, curved claws facilitating food excavation. The fourth digit of the hind limb is enlarged with the smaller fifth digit acting as the main weight bearing structure (Blyde 1999). The second and third digits are small and syndactylous. The first digit is absent.

Females possess a pouch which is supported by paired epipubic bones. They also have paired lateral vaginae and a median vagina. The reproductive tract lies laterally to the urogenital tract. At parturition, a birth canal is formed in the connective tissue between the median vaginae and the urogenital sinus through which the fetus passes (Blyde 1999). Like all macropods, potoroos have a cloaca. In the females the rectum is dorsal to the urogenital

opening while in the males the rectum is dorsal to the penis (Tyndale-Biscoe and Renfree 1987).

Gilbert's potoroos do not appear to have a defined breeding season, with wild pouch young (> 30mm crown-rump length) being recorded throughout the year, (Courtenay and Friend 2005). Captive-bred young have been born in February (n=1), April (n=2), August (n=1) and December (n=3). Oestrous length has been estimated between 41-43 days in one captive female (T. Fletcher, personal communication, March 20, 2005). The oestrous cycle of other species of potoroids tends to last 20-30 days, while the macropodoids typically have cycles from 30-45 days (Jackson 2003).

Young in captivity have first been observed out of the pouch at 150g in body weight, achieving permanent pouch exit when approximately 190g (Courtenay and Friend 2004). Following emergence from the pouch, juveniles gain 6g on average within the first few months. Only one young is born at a time. The only species of macropod routinely producing twins, and sometimes triplets, is the musky-rat kangaroo (*Hypsiprymnodon moschatus)* (Dennis 1997).

Females exhibit embryonic diapause, whereby reactivation and development of a quiescent blastocyst can occur. One wild female brought into captivity subsequently lost her pouch young, yet produced a new young without being in contact with a male (Courtenay and Friend 2004). In November 2005 another wild caught female with a male furred pouch young was brought into captivity for translocation. The pouch young was removed for hand rearing and within two weeks another pouch young was found on physical examination. There had been no contact with males from the time of bringing the animal into captivity to translocation. As such, it would seem theoretically possible for female Gilbert's potoroos to have three young at varying stages of development. One in a state of embryonic diapause, one in the pouch attached to the teat and one which has vacated the pouch permanently,

returning to the pouch only to feed (Jackson 2003). The neonate in embryonic diapause is born shortly after the joey in the pouch permanently vacates the pouch. This phenomenon is also exhibited in other marsupials, including kangaroos (Blyde 1999).

Sexual maturity in Gilbert's potoroos is estimated to be twelve months in females (Courtenay and Friend 2004). A captive male of known age was thought to have sired young at 14 months of age, yet sperm was only detected in urine of another captive male at two years of age. These figures are similar to the long-nosed potoroo which can remain reproductively active in captivity for up to seven years (Ullman and Brown 1983). The maximum age at which a captive male Gilbert's potoroo has mated is six years. The maximum age at which a captive female has given birth is five years. However, a wild female estimated to be seven years in age has been found to have a young at heel (Courtenay and Friend 2004).

It has been proposed that Gilbert's potoroos exhibit a monogamous mating system. Several Gilbert's potoroos paired with individuals in captivity did not produce young, although these individuals had successfully reproduced with other mates in captivity (Courtenay and Friend 2004). This is similar to the mating system proposed for the long-footed potoroo (Scotts and Seeback 1989) but is considerably different to the polygamous mating system reported in the long-nosed potoroo (Jarman 1991). However, Long (2001) found long-nosed potoroos at the Ralph Illidge Sanctuary in south-western Victoria to change from polygamy to monogamy depending on the ability of the male to locate and monitor female sexual status.

Seeback and Rose (1988) state that 'Potoroos are generally long lived with life spans of more than seven years in the wild and up to 12 years in captivity'. The oldest Gilbert's potoroo recorded in captivity was ten years old, while the oldest wild Gilbert's potoroos found have been estimated to be over seven years of age (both these estimates being derived from tooth eruption and wear) (Courtenay and Friend 2004). Seeback (1982)

reported the average life expectancy of the captive long-nosed potoroo to be between four to five years, although one individual survived for up to nine years. Courtenay and Friend (2004) estimate the average life expectancy of the captive Gilbert's potoroo is similar to this figure.

Gilbert's potoroos can be divided into broad age categories on the basis of tooth eruption (in younger animals) and tooth wear (in older animals). However, tooth wear patterns would be expected to differ amongst the wild and captive population, reflecting the differences in diet and the amount of grit ingested (Courtenay and Friend 2004). The deciduous dental formula constitutes three incisors, the diastema then a deciduous pre-molar and five molars. The permanent adult dental formula comprises 3 incisors, the diastema then the premolar (which replaces the deciduous pre-molar and M1) followed by four molars, M2, M3, M4 and M5. (Courtenay 2000). Young at pouch exit (roughly four months of age, at approximately 190g in body weight) typically have deciduous upper and lower central incisors, the deciduous premolar and M1 with the second deciduous incisor and M2 erupting. The deciduous canine begins to erupt just prior to weaning, at six to nine months of age, at an approximate 450g in body weight. At 12 to 14 months, at an approximate weight of 875g, the permanent premolar should be seen to erupt, as should M4 and the permanent canine and I2. Finally at 14 to 23 months, M5 should erupt (Courtenay 2000).

Courtenay (2000) reports that tooth wear is most commonly observed on anterior teeth and on the lingual cusps. While individual variation exists, cusps have been found to wear most commonly from the anterior lingual to the posterior lingual, and from the anterior buccal to the posterior buccal. From the pattern of cusp wear, age correlations have been made. However, Courtenay (2000) emphasizes the importance of taking the potoroos' reproductive condition and other age related changes such as arthritic joints, cataracts and greying of the fur around the muzzle and ears, (as seen in animals older than six years) into account.

1.6.2 Reproduction

Captive breeding by conventionally pairing animals in pens initially seemed successful, with the production of eight offspring from 1994 to 2001. However, following this, the rate of production of young diminished, with no young being recorded after February 2001. Faecal hormone studies (Stead-Richardson *et al*. 2005) showed that captive females were cycling, and sperm was present in male urine. Attempted matings (recorded through videotape surveillance) occur frequently, yet three-weekly pouch checks reveal a constant absence of pouch young (Friend 2005). Attempts at assisted reproduction and fostering have been undertaken to try to increase the reproductive success of this species, but have had limited success. The cross-fostering of Gilbert's potoroo pouch young into long-nosed potoroos in South Australia is one example. In the 1960s Merchant and Sharman (1966) discovered that marsupial pouch young attached to the teat can be transferred from one pouch to another. It was thought that the removal of pouch young from the teat would lead to cessation of the suckling stimulus. This would either then reactivate the development of a quiescent blastocyst (if present), or cause the animal to return to oestrous, mate and produce another young and so increase the reproductive rate (Courtenay, unpublished data). This technique is being used in the Victorian brush-tailed rock-wallaby (*Petrogale penicillata)* conservation program to maximise the female reproductive rate and to accelerate the production of young (Taggart 2002). Cross-fostered young of several species, including brush-tailed rock-wallabies and brush-tailed bettongs (*Bettongia penicillata)* have also been raised to maturity and bred in captivity.

Trials of cross-fostering in a range of potoroid species (long-nosed potoroos, and brush-tailed bettongs) were carried out in South Australia by Dr David Taggart in order to select a suitable surrogate species for the Gilbert's potoroo. These trials indicated that long-nosed potoroos would be the most suitable species for fostering Gilbert's potoroo young (Friend 2006).

The cross-fostering research program involved the removal of a suckling Gilbert's potoroo pouch young and transferal to the pouch of a surrogate long-nosed potoroo female, which had a pouch young at a similar stage of lactation. Three attempts at this procedure were undertaken. All three survived the ten hour pouch isolation associated with transportation from Albany in Western Australia, to Adelaide in South Australia where the long-nosed potoroos were housed. However, the Gilbert's potoroo pouch young only survived for between one week and one month following transportation. Reasons for this may include the ten-hour pouch isolation, and that two of the three lactating long-nosed potoroo females were transferred from large outdoor runs into small holding enclosures on the same day that the cross-fostering procedure was undertaken. This sudden change in circumstances may have led to stress in the surrogate females, which could have adversely affected their milk-producing potential and general health.

In 2006, a long-nosed potoroo colony was established in farmland adjacent to the Two Peoples Bay Nature Reserve for cross-fostering purposes. This meant that pouch transfers could be carried out within minutes of removal of pouch young from the wild. The initial trial in March 2007 was unsuccessful. Another trial commenced in February 2008 currently seems successful (T. Friend, personal communication, February 20, 2008).

Other aspects of on-going reproductive research include hand-rearing furred young from wild females to produce animals that are habituated to handling. These animals may be of use in future captive breeding and cross-fostering initiatives. Transfer of the captive population to 5-20 hectare fenced enclosures has also been proposed as a means to improve captive breeding success by potentially reducing stressors associated with co-habiting in small enclosures in captivity. Similar methods have been used to breed other marsupial species in Western Australia at Heirisson Prong (Shark Bay) and in the *Return to Dryandra* project. Two captive potoroos were transferred into a large fenced enclosure in December 2007 (T. Friend, personal communication, December, 2007). However these

methods are all unproven in the Gilbert's potoroo and are seen as medium to long-term strategies.

Another area of assisted reproduction being investigated is artificial insemination (AI). Attempts have been made at Perth Zoo, to collect semen samples from long-nosed potoroos. The aim of this research was to refine the procedure in the long-nosed species before the procedure was attempted in the Gilbert's potoroo. AI has been developed for several species of marsupial (Molinia *et al.* 1998, Johnston *et al*. 2000, Paris *et al*. 2002) with intrauterine injection of sperm being the most successful technique. AI for marsupials is a relatively recent development and is hampered by the lack of suitable procedures for freezing sperm (Fletcher, 1997, unpublished data). This means that fresh sperm must be collected from a male at the optimal time for insemination of the female and that the stage of the oestrous cycle in a female must be readily determined. Magarey (2000, unpublished data) conducted preliminary studies in oestrous detection in long-nosed potoroos using cytology of the reproductive tract and examination of the changes in proportions of partly cornified epithelial cells in smears taken from the anterior urogenital sinus. Stead-Richardson (1999, unpublished data) also conducted blood progesterone analysis in order to define the oestrous cycle of this species. A less stressful method of monitoring oestrus using faecal hormone analysis is available, but the time lag between changes in hormone secretion and their detection via faecal metabolites is a disadvantage, (Stead-Richardson 2005). The use of teaser males was also suggested by Magarey (2002, unpublished data). Identification of methods for oestrous detection requires further investigation.

Initial attempts at collection of sperm from long-nosed potoroo males at Perth Zoo had limited success and resulted in small volumes of semen being collected. The difficulty associated with semen collection, combined with the lack of regular cycling in the females and lack of funding has meant the program has been temporarily put on hold.

Temporary translocation to a predator free offshore island, known as Bald Island was trialed for five weeks using a male and female sub-adult from Mount Gardner in the winter of 2005 (Friend 2006). Following the successful trial, these animals were retrieved and retained in captivity. In August 2005 these two individuals and another male and female were permanently translocated to Bald Island. In April 2008, seven potoroos were caught, and the first female to be born on the island was found to have pouch young. This was the first evidence of a second generation on the island. The first female taken to the island also has pouch young and a joey at heel (T.Friend, personal communication, May 1, 2008).

1.6.3 Nutrition

A detailed study of the diet of wild Gilbert's potoroos is underway. The Gilbert's potoroo has the most specific diet of any species of potoroo. A study of the dietary composition based on faecal analysis (Nguyen 2000) found that fungal material made up more than 90% of the diet. While 44 species of fungi were consumed in total, five species were preferred by over 60% of animals in all seasons of the year. This study provided details of spore types found in potoroo faeces but further investigation is needed to link these spore types with species of truffle-like fungi identified in the field (Courtenay *et al*. 2002). This will enable the formulation of a captive diet to closely match the nutrient profile of the wild species, and to eliminate dietary inadequacy as a potential reason for the lack of reproductive success. Currently, regular collections of these truffle-like fungi are made in forestry plantations near Two Peoples Bay and the captive diet now consists of 10% hypogeal fungi (by weight) (Courtney and Friend 2004).

1.6.4 Habitat

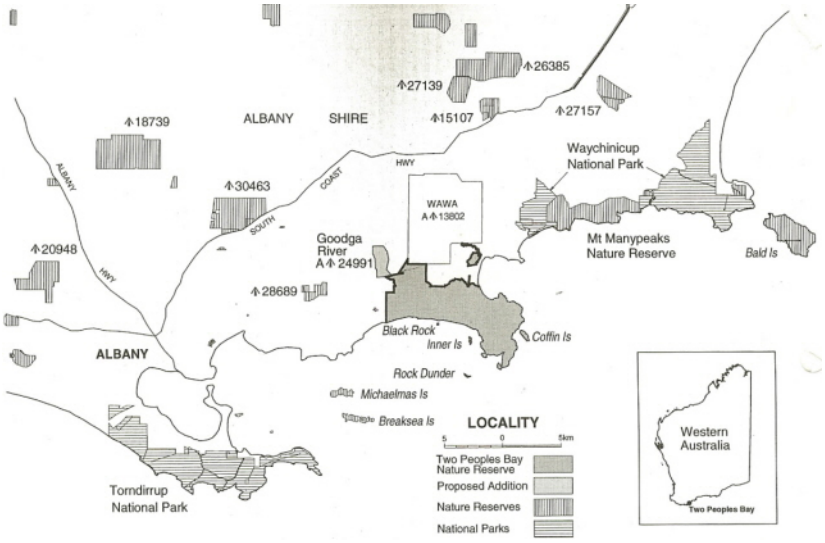
The Gilbert's potoroo is found within 1000 hectares of heath land in the valley slopes of Mount Gardner in Two People's Bay Nature Reserve (Figure 1.1). The Two Peoples Bay area is 35km east of Albany and was classified as an 'A' Class Nature Reserve for the

Conservation of Fauna in 1967 as a result of the 1961 rediscovery of the Noisy Scrub-bird (*Atrichornis clamosus*) thought to be extinct. The Two Peoples Bay area is considered to be the most significant area for endangered birds in mainland Australia (Garnett 1992). Other threatened birds sharing this Nature Reserve include the major populations of the Western Bristlebird (*Dasyornis longirostris*) and the Western Whipbird (*Psophodes nigrogularis leucogaster*) (Orr *et al.* 1995, WA Wildlife Conservation Act).

The Gilbert's potoroo occupies dense scrub and heath vegetation within the reserve. Dominant species within the reserve include 1.5 to 2m *Melaleuca stratia* and *Melaleuca uncinata*, with dense layers of the sedges *Lepisoderma spp*. and *Anarthria scarbia* being found beneath (Courtney and Friend 2004). This area has remained unburnt since the 1960's. It is considered that long unburnt vegetation is required to support the Gilbert's potoroo (Courtenay and Friend 2004).

The potoroo shares the Nature Reserve with a recorded 28 mammalian species, including marine mammals found on offshore islands which are also part of the reserve. Twelve of these species are marsupials, ten are eutherians and six are introduced species (Orr *et al.* 1995). Large numbers of western-grey kangaroos *(Macropus fulginosus)* are found within the reserve. The quokka (*Setonix brachyurus*), which is found in low numbers on the mainland also inhabits the area, as does the brush wallaby (*Macropus irma*), the yellow footed antechinus (*Antechinus flavipes*), the southern-brown bandicoot (*Isoodon obesulus),* the western-ringtail possum (*Pseudocheirus occidentalis*), and the honey possum (*Tarsipes rostratus*). Of these, only the southern-brown bandicoot and the western-ringtail possum have been declared threatened (WA Wildlife Conservation Act).

Figure 1.1 Locality of Two Peoples Bay Nature Reserve



Thirty-four species of herpetofauna have been recorded within the reserve, including seven snakes, 13 skinks, one gecko, two legless lizards, one monitor, one tortoise and nine frogs (Orr *et al.* 1995). Of these, only the carpet python *(Morelia spilota imbricata)* has been declared in need of special protection by the WA Wildlife Conservation Act. This may be owing to its propensity to bask on roads within the reserve.

Four threatened species of flora are also found within the reserve (Courtenay and Friend 2004).

Much of the reserve is affected with dieback disease, *(Phytophera cinnamoni)* caused by an introduced soil-borne fungus, which appears to have been present for at least 40 years (Orr *et al.* 1995). Dieback is spread through the movement of fungal spores in water and moist soil. It is thought that the disease was originally introduced through infected gravel used to make the Two Peoples Bay Road, and once present in corridors may have been transmitted by kangaroos following trails linking non-infected and infected areas. The disease was then perpetuated by a wet climate and soil types favouring transmission. The presence of a

number of vegetation species that are highly susceptible to disease also facilitated its spread.

Dieback has altered the structure of vegetation over time. It is currently affecting *Hakea* and *Dryandra* dominated scrub and thicket on Mount Gardner leaving a more open sedge-dominated community (Orr *et al.* 1995). Potoroos are currently thought to be present only in areas free of infection. However, given that their major feed source is mycorrhyzal fungi, destruction of host species from disease and vegetation changes could have far reaching effects (Courtenay and Friend 2004).

Current management techniques to limit further spread of dieback in the reserve include washing down vehicles entering and leaving infected areas, and brushing down footwear when walking through infected areas.

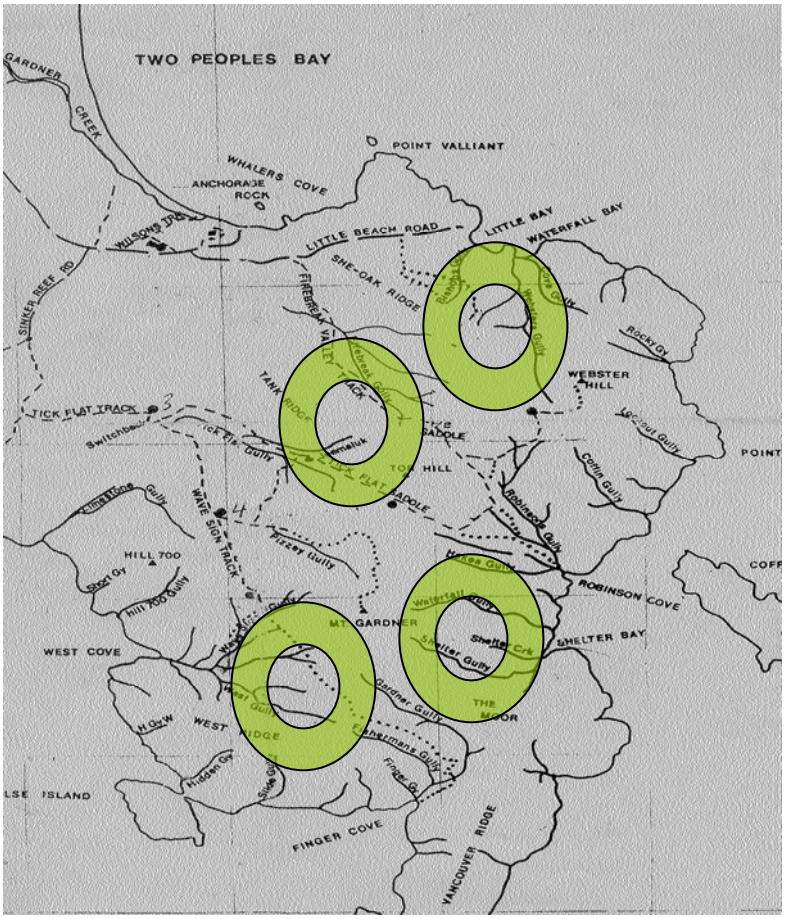
1.6.5 Spatial dynamics

The Gilbert's potoroo has an extent of occurrence estimated to be 8km^2 and an area of occupancy estimated to be less than 5km^2 on Mount Gardner in the Two Peoples Bay Nature Reserve. Within the nature reserve the majority of potoroos can be found within four distinct areas (Courtenay and Friend 2004) (Figure 1.2).

Trap lines have been established in these localities subject to accessibility. Every March, July and November for three, four-day periods traps are set on a rotational basis as outlined in the Gilbert's Potoroo Recovery Plan. This time frame was chosen as it was thought to enable re-trapping of pouch young with their mothers, which would provide data on development and the length of time spent in the pouch (Courtenay and Friend 2004). Trapping effort increased in 1999 to 2001 with a greater number of traps being set over a larger range. From 2001 onwards the trapping effort stabilized to the current level which involves trap lines containing between five to 20 Sheffield cage traps. These three, four-day

trapping rounds include the North, South, East firebreak and Upper Robinson's trapping lines, the Hakea and West-6 trapping lines, and Bishop's Gully and the Waterfall trapping line. It is thought that the majority of potoroos in the population have access to these trapping lines.

Figure 1.2 The majority of Gilbert's potoroos are found within four distinct localities on Mount Gardner.



The number of potoroos regularly trapped has increased from eight in 1999, to 17 in 2003 (Courtenay and Friend 2004). This is thought to be a reflection of increased trapping effort rather than population growth. The current population is estimated to be approximately 35 individuals (T. Friend, personal communication, February 20, 2008). A population study has shown that on average, 10 pouch young are produced each year in the trapped area, but that at most four survive to adulthood (Friend 2006). Both young and old animals disappear

from the trapping record, but are generally replaced by new young animals. Friend (2006) describes the population as robust but small.

Trapping and radio-tracking have shown that Gilbert's potoroos live in small groups within the reserve area. These groups exist as separate colonies however, older males and dispersing sub-adult males may co-habit multiple colonies. Potoroos of the same sex tend not to overlap in home-range while males and females may overlap (Courtenay and Friend 2004) Male Gilbert's potoroos have been estimated to have a home range between 15-25 hectares whilst females, young at heel and dispersing sub-adults of both sexes tend to have a smaller range of between 3-6 hectares (Friend 2000).

1.6.6 Previously reported disease in the Gilbert's potoroo

Previous investigation of health problems in this species have been conducted by Drs Anne-Marie Horwitz, Kevin Ellard, David Forshaw and the veterinarians at Perth Zoo. The following disease issues of captive Gilbert's potoroos have been identified; renal oxalosis with glycolic aciduria, disseminated *Mycobacterium intracellulare* infection, *Cryptococcus neoformans* encephalomyelitis, *Chylamydophila*, proctitis associated with a *Treponema*-like organism, squamous cell carcinomas of the teat, a malignant sarcoma, a skin haemangioma, erythroid myelosis, *Chytostethum* mite infestation, an intestinal *Potorostrongylus* nematode infection, *Ophidascarsis* larval nematode infection of body cavities and viscera, *Brienlia* larval nematode infection of body cavities and an unidentified nematode infection of the brain. No routine testing had been undertaken for *Toxoplasma* infection in the wild population.

The results of the previous investigations are outlined below, and some of the disease processes mentioned will be discussed further in specific chapters.

1.6.6.1 Renal oxalosis

Severe renal oxalosis has been the cause of death in five animals in the captive population (D. Forshaw, personal communication, November 20, 2007). Renal oxalosis refers to the microscopic deposition of oxalate in the tubules causing tubular epithelial necrosis and dysfunction (Moffat 1977). Two of the five potoroos also had uroliths. Uroliths are rock like collections of minerals found in the urinary tract (Radostits *et al.* 2000). Uroliths may cause urinary tract mucosal damage and subsequent bacterial infection, and if of an appropriate size can obstruct urine outflow leading to post-renal azotaemia. Total outflow obstruction results in acute renal failure and death (Bryant 2003). No associated signs of obstruction or infection were present in the potoroos (D. Forshaw, personal communication, November 20, 2007).

It is interesting to note that four of these deaths have occurred in one family group. This high incidence is highly suggestive of an inherited condition (Horwitz and Forshaw 2001, unpublished data). Testing conducted by Dr Barry Lewis at Princess Margaret's Hospital in Perth, Western Australia, demonstrated large amounts of glycolate in the urine of potoroos with renal oxalosis.

Generally hyperoxaluria occurs due to increased production of metabolic oxalate (heritable disorders) (Danpure *et al.* 2001), increased intake of oxalate containing foodstuffs (especially herbage) (Radostits *et al.* 2000), inappropriately increased intestinal uptake of dietary oxalate (secondary to enteric disease) (Smith 1990) or inappropriately increased renal tubular excretion of oxalate (Gambaro *et al.* 1995).

Excess dietary oxalate intake was eliminated as a possible cause of the renal oxalosis (D. Forshaw, personal communication, November 20, 2007) and at present, a probable inherited disorder of oxalate metabolism involving the enzyme pathways around the intermediary substrate glyoxylate (which is degraded to oxolate and glycolate) and enzyme

mistargetting is the most likely explanation (Horwitz and Forshaw 2001, unpublished data). However, the possibility of other unprecedented mechanisms cannot be dismissed.

In humans, all abnormalities of the enzyme alanine-glyoxylate aminotransferase (AGT) resulting in hyperoxaluria are inherited as an autosomal recessive mode of inheritance. However, currently there is not enough evidence to confirm this mode of inheritance in the potoroos (Horwitz and Forshaw 2001, unpublished data). Dr David Forshaw of DAFWA has been investigating the mechanism of biochemical changes in the potoroos in conjunction with Professor CJ Danpure in London.

In May 2007 a male Gilbert's potoroo pouch young was cross-fostered into a long-nosed potoroo, however subsequently died following cross-fostering when returned as a sub-adult to the captive colony. This individual was found to have severe, acute, multifocal renal tubular necrosis, with probable oxalate crystalluria and a severe, acute, diffuse balanoposthitis (D. Forshaw, personal communication, November 20, 2007). The acute renal tubular necrosis was presumed to result from toxicosis or ischaemia due to hypoperfusion of the kidney as seen in circulatory shock. However, the presence of moderate numbers of oxalate crystals in the kidney raised the possibility of potoroo hyperoxaluria syndrome (PHS). Renal oxalosis can be seen in other conditions and, animals that have died of PHS in the past have had more severe renal changes. Therefore it is possible that the acute tubular changes seen were superimposed on a developing renal oxalosis (D. Forshaw, personal communication, November 20, 2007). The lack of urine in the bladder precluded testing for oxalates or glycolate, the interval from death to discovery precluded blood sample analysis for renal function and there was insufficient ocular fluid to run renal function tests. The dam of this animal was suspected to be a carrier of the disease.

Oxalate urolithiasis is a relatively common complaint in humans and domestic carnivores yet is infrequently reported in marsupials. Bryant (2003) reported four cases of calcium oxalate

urolithiasis in captive macropods, the red-kangaroo *(Macropus rufus),* a western-grey kangaroo, a red-necked wallaby *(Macropus rufogriseus banksianus)* and an eastern-grey kangaroo *(Macropus giganteus giganteus).* These cases highlighted both obstructive and incidental calcium oxalate urolithiasis. It was unknown whether the uroliths were formed as a result of hyperoxaluria, hypercalciuria or other factors.

A case of oxalate poisoning in a koala *(Phascolarctos cinereus)* was reported by Canfield and Dickens (1982). The source of the oxalate was not determined, and no other con-specifics were affected. Histologically the kidney tubule lumens were filled with crystals and stained positively when treated with Pizzolato's peroxide silver method for calcium oxalate (Pizzolato 1964). While the prevalence of oxalate toxicity in koalas is undetermined Canfield and Dickens (1982) commented that the prevalence of renal oxalosis may be higher than reported.

While an inherited disorder of oxalate metabolism seems the most likely cause of the potoroo renal oxalosis syndrome, (D. Forshaw, personal communication, November 20, 2007) husbandry changes have been initiated to minimise crystalluria in the captive potoroo population. Oxalate-containing feed items are avoided, and food items high in vitamin D and calcium are also limited, as such diets may predispose marsupials to hypercalciuria and the subsequent development of calcium oxalate urolithiasis (Bryant 2003). Water is available *ad lib* to maintain urine output and urinalysis is performed opportunistically to check glycolate levels.

1.6.6.2 Atypical mycobacteriosis

A diagnosis of *Mycobacterium avium-intracellulare* was made on post mortem examination of Gilbert's potoroo male six (GP M6) in January 2001. This potoroo had been euthanased after failing to respond to treatment of progressive disease (Horwitz and Forshaw 2001, unpublished data). Initial presenting signs in the five year old male included collapse, recumbency, anorexia, and weight loss. Histologically, a disseminated granulomatous

inflammatory disease was present with large numbers of acid-fast bacilli in necrotic lesions in the spine, liver and lung. This potoroo also had bilaterally enlarged adrenal glands which may have related to the disease process itself, or to a sign of stress, which could lower immunity (Horwitz and Forshaw 2001, unpublished data).

Atypical mycobacteria such as *M. avium* complex are ubiquitous in the environment and most commonly found in the faeces of domestic animals, wild birds, soil, as well as dust and water (Wolinsky 1988). This makes control or elimination of these acid-fast bacilli from contaminated premises difficult (Thoen 1993). In marsupials, lesions are typically localized to the lungs, skin and bone (Joslin 1990) suggesting inhalation of infectious aerosols, inoculation due to bite and deep puncture wounds or haematogenous dissemination.

Marsupials appear to be highly susceptible to atypical mycobacteriosis, (Thoen *et al.* 1978, Mann *et al.* 1982). *M .avium* has been reported in a number of captive marsupials including the brush-tailed bettong, the tree-kangaroo *(Dendrogalus matschiei)*, and the long-footed potoroo, (Buddle and Young 2000). An earlier report by Lesslie (1958) also reported atypical mycobacterial disease in the red-necked and Bennetts wallabies (*Macropus rufogriseus* and *bennetti*), the rufous rat-kangaroo (*Aepyprymnus rufescens*), the Kangaroo Island kangaroo (*Macropus fuliginosus*), and the black-faced kangaroo (*Macropus giganteus melanops*). Gaynor and Friend (1990) also described infection and clinical disease in numbats (*Myrmecobius fasciatus*).

The observed susceptibility to atypical mycobacterial disease in marsupials has been proposed by Buddle and Young (2000) to involve deficiencies in cellular immunity of the marsupial immune response. In examining numerous histological samples Buddle and Young (2000) concluded that marsupials, unlike eutherians, seemed unable to wall off and localize disease, leading to the formation of satellite lesions and generalised disease.

Recovery of marsupials from mycobacterial infections is rarely reported (Buddle and Young 2000).

In the captive colony of long-footed potoroos at Healesville sanctuary in Victoria, Australia, *M. avium-intracellulare* is a recurring problem. Their ground dwelling nature, foraging activity, and access to bird faeces may further increase their susceptibility to disease. From January 1986 to February 1996, 18 mortalities occurred. Five of these deaths were directly attributed to *Mycobacterium avium-intracellulare*. Phelan (1996) reported that two of these cases were diagnosed on necropsy and the remaining three were diagnosed by recovery of acid-fast bacilli from tracheal washes and fine needle aspiration in potoroos presenting with signs of respiratory disease. Chemotherapy with a combination of rifabutin, ethambutol and clarithromycin was trialed without success.

The diagnosis of mycobacterium in the captive colony of Gilbert's potoroos prompted the spelling of GP M6 enclosure for three years and 10 months. The sand in the front of the enclosure, under the shelter, was changed, but the remaining two-thirds of the cage comprising peppermint and shrubbery which is open to the weather was not removed. Quarterly sand changes of substrate have now been invoked to help minimise the environmental load of mycobacteria in the captive potoroo enclosures.

While only one death has been attributed to mycobacterial disease in Gilbert's potoroos their susceptibility as a marsupial species and their ground dwelling nature has prompted this disease to remain as a differential diagnosis in potoroos presenting with respiratory signs.

1.6.6.3 Cryptococcosis

Dr Mark Krockenberger of the University of Sydney confirmed a case of *Cryptococcus neoformans var. neoformans* in GP M7 in September 2000. This animal had been euthanased after a progressive illness that was non-responsive to treatment. A post

mortem diagnosis of meningoencephalitis had been made (Horwitz and Forshaw 2001, unpublished data). This disease can be contracted by aerosol inhalation and was presumed to be spread via faecal contamination from Bronzewing pigeons (M.B. Krockenberger personal communication, July 20, 2004). Cryptococcosis is seen in immunosuppressed individuals although it can also be found in normal individuals. This disease will be discussed in detail in Chapter four.

1.6.6.4 Chlamydophila

A *Waddlia chondrophila* was detected on a cloacal swab in 2003 from a potoroo in the captive-breeding colony. This animal did not have evidence of clinical disease (Bodetti *et al.* 2003). Chlamydiales cause a wide range of disease in a wide variety of marsupial hosts and can result in a range of conditions from inapparent sub-clinical infection through to severe clinical disease (Bodetti *et al.* 2003).

1.6.6.5 Balanoposthitis

A long history of inflammation of the penis has been reported in both the wild and captive population of Gilbert's potoroo (Horwitz and Forshaw 2001, unpublished data). However, the clinical significance of infection was unknown. Crusty preputial exudates were seen in both the wild and captive population, and the only treatment offered involved cleaning the prepuce of severely affected animals with antiseptic solution (Horwitz and Forshaw 2001, unpublished data). In August 2001, the founder captive individual GP M3 was found to have a bright green discharge. Microscopically this discharge contained large numbers of spirochaetes. Treatment with penicillin seemed to resolve the infection (Horwitz 2001, unpublished data). A *Treponema*-like organism was also identified via PCR from preputial and cloacal swabs from the male and female Gilbert's potoroo transferred to Perth Zoo. The infection in the male, in conjunction with a superficial exudative dermatitis failed to respond to topical and systemic antibiotics, and this animal later died. It is possible that the frequent handling for treatment may have been a factor in its demise (C. Monaghan, personal

communication, February, 2002). This clinical entity will be discussed in detail in Chapter six.

1.6.6.6 Pouch masses

Squamous cell carcinomas were identified via histopathology in the pouches of two captive female potoroos (GP F4 and GP F10). Another female has a mass, which is yet to be excised. The possibility of an inherited predisposition to the tumor given its high prevalence in such a small population, or the possibility of a common environmental teratogen has been considered (Horwitz and Forshaw 2001, unpublished data).

Apart from papillomas associated with pox virus, neoplasms are uncommon in free-ranging macropodidae (Speare *et al*. 1989). This is quite different to the dasyurids which seem highly susceptible to tumour development. Only one report of neoplasia was found in macropods, that of a well differentiated squamous cell carcinoma in the stomach of a wild red-bellied pademelon (*Thylogale billardierii),* in Tasmania (Munday 1971). Within the carcinoma large numbers of *Labiostrongylus* spp. larvae were found with surrounding hyperplasia and mucosal nodules (Speare *et al.* 1983). Munday (1971) proposed the neoplasia to have occurred as a result of chronic irritation incited by the parasite leading to neoplastic transformation. However in other marsupial species, including the swamp wallaby (*Wallabia bicolour),* no evidence of neoplasia associated with lesions induced by *Labiostrongylus* was found (Beveridge, Presidente and Speare 1985).

1.6.6.7 Skin lesions

One captive-born potoroo, GP M46 developed crusty skin lesions due to the presence of the microscopic mite, *Cytostethum spp.* in the Family Atopomelidae. This mite has been detected in wombats without clinical effect (Booth 1999). This infestation was presumptively treated with the macrocyclic lactone Ivermectin but little success was noted (Horwitz, 2001 unpublished data).

1.6.6.8 Malignant sarcoma

An aged female wild potoroo in poor body condition was found dead in June 2004 possibly following regurgitatation by a carpet python. Multiple nodules in the liver, kidney and lung were found resembling an undifferentiated sarcoma. Forshaw (2007, unpublished report) commented that there was differentiation towards a haemangiosarcoma in some sections, accompanied by significant haemorrhage into the tumour masses. However, the aetiology was unknown. This individual also had concurrent intestinal helminthiasis caused by *Potostrongylus temperatus*, *Ophidascaris robertsi* infestation of the pleural and peritoneal cavities; as well as *Breinlia* spp., (possibly *B macropi*) infestation of the pleural and peritoneal cavities. *Breinlia macropi*.sp. has been previously described to affect the liver of the quokka (Wahid 1962).

1.7 THE FOCUS OF THE GILBERT'S POTOROO HEALTH MONITORING PROJECT

This research project was undertaken over a three year period and involved long-term health monitoring of wild and captive populations of Gilbert's potoroo in the south-west of Western Australia.

The research aimed to determine the prevalence of specific diseases in the wild and captive population and to test for correlation with the effects of identified diseases on population dynamics including reproductive success and survivorship. The study identified whether infections associated with pathogenic organisms that resulted in the death of potoroos in the captive-breeding colony, were also present in the wild population.

The study encompassed retrospective analysis of captive health records and trapping data collected during the study period and statistical analysis of this data to determine if there was any association between overall health status, reproductive success and survivorship.

It is interesting that many of the diseases associated with the deaths of captive potoroos generally result in sub-clinical infection and so the possibility of underlying immunosuppression was also considered. Diseases such as toxoplasmosis, chylamydophilosis, salmonellosis, cryptococcosis and gastrointestinal parasite infections can be fatal in animals that have compromised immune function, (Attwood *et al.* 1975; Laitinen *et al.* 1996; Carter and Wise 2004; Helke *et al*. 2006; Sleeman *et al.* 2000) and could cause serious reductions in the size of small populations of threatened wildlife species (Daszak *et al.* 2000). Potentially immunosuppressive retroviruses have also been detected in long-nosed potoroos and other marsupial species, and stress itself in the form of handling, dietary, environmental and social structure change may also result in immunosuppression.

The prior identification of a *Treponema*-like organism in the Gilbert's potoroo is potentially of great importance. However, this organism had only been sequenced from samples from one potoroo and its pathogenicity has yet to be determined. To determine the clinical significance of this micro-organism, this epidemiological study involved whole population sampling for the *Treponema*-like organism, in combination with physical examination for evidence of balanoposthitis and other reproductive tract abnormalities.

The findings of this research project will facilitate the formulation of management protocols to minimise the risk of these disease processes impinging upon a genetically bottlenecked species.

1.7.1 Aims of the health monitoring project

i) To study and determine the general health of all individuals in the captive colony and a sample of the wild population by a variety of diagnostic methods, including:

- Assessment of body condition and weight
- General physical and ophthalmological examination
- Haematology and biochemistry
- Urinalysis
- Serological testing for toxoplasmosis via the Direct Agglutination Test (DAT) and the Modified Agglutination Test (MAT)
- Serological testing for cryptococcosis via the Latex Cryptococcal Antigen Test (LCAT)
- Cloacal swabbing for *Salmonella* culture and typing if present
- Feet and nasal swabbing for *Cryptococcus* culture
- Preputial and cloacal swabbing for identification of *Treponema*-like organism via PCR assay
- Collection of faecal samples from traps and enclosures for faecal flotation examination for parasites
- Skin scrapings - for ectoparasites
- Soil sampling for *Cryptococcus* organisms
- Post mortem examination of any captive individuals in the breeding colony that die

ii) To formulate haematological, biochemical and urine reference ranges and evaluate these reference ranges in light of health and disease status (Appendix 13 and 14).

iii) To assess and determine the prevalence of a *Treponema*-like organism in the captive and wild potoroos, involving preputial and cloacal swabbing and PCR analysis.

iv) To study and compare the reproductive success and causes of mortality in captive bred and wild potoroos, involving retrospective analysis of captive health records and, analysis of disease testing and trapping data collected during the proposed study.

v) To formulate population health management procedures to facilitate on-going management of the wild and captive population.

1.7.2 Significance of the health monitoring project

The threat posed by infectious diseases to the long-term survival prospects of critically endangered species has been well documented (Daszak and Cunningham 1999, Daszak *et al.* 2000, Daszak *et al.* 2001, Sims 2001, unpublished data). The potential for disease to drive population decline and species extinction is a possibility that cannot afford to be discounted, and one that must be addressed in any endangered species recovery plan.

In examining past necropsy and veterinary clinical reports from the Gilbert's potoroo captive breeding project there are a number of specific disease processes evident which require further in-depth investigation, analysis and surveillance. The health status of the remaining two potoroos in captivity and the estimated 35 wild individuals needs to be determined to ensure that any potential disease risks are identified, to enable the potential for translocation to be fully explored and to maximise the likelihood of success of the Gilbert's potoroo recovery program.

This study aims to make an important contribution to the veterinary management of the critically endangered Gilbert's potoroo. The collection and analysis of data in this study will also provide a critical approach to disease risk assessment associated with management of captive and wild populations of endangered species, and serve as a model upon which the captive management and translocation of other endangered species can be based. Furthermore, this study will form and pave the way for further studies in the newly emerging field of Conservation Medicine.

1.8 CHAPTER ORGANISATION

A general materials and methods chapter follows this introduction, discussing trapping protocols and general anaesthesia, materials and methodology used to collect samples.

The next three chapters, comprising Chapter's three to five focus on the primary disease conditions affecting Gilbert's potoroos. These chapters begin with a literature review looking at the importance of the particular disease in relation to other mammals and in particular marsupials. Specific materials and methods required for the study of these diseases in the Gilbert's potoroo are then discussed. Results are demonstrated and displayed, and a discussion follows. Chapter five discusses the microbiology of the cloaca in a similar format to the preceding chapters and reports on commonly isolated species from both the rectal opening of the cloaca, and the urogenital opening of the cloaca. Chapter six discusses *Treponema* infection, its significance and potential pathogenicity in the Gilbert's potoroo in a similar format to the above.

Chapter seven comprises a general discussion of all the results obtained and places these findings within the context of the health, reproductive success and survivorship of the Gilberts potoroo population. This chapter draws together all the results and discussions and proposes a translocation protocol to minimise any current or potential disease impact on the Gilbert's potoroo population.

CHAPTER 2

General materials

and methods

2.1 TRAPPING AND SAMPLE COLLECTION REGIME

2.1.1 Trapping regime

Wild potoroos are trapped three times per year (March, June and November) by DEC staff to monitor the population in accordance with the Gilbert's Potoroo Recovery Plan ratified by the Gilbert's Potoroo Recovery Team.

This study involved the trapping and examination of between four and six individuals from the captive colony, and approximately 20 individuals from the wild population, three times a year in 2005, twice in 2006 and once in 2007 (Table 2.1).

Table 2.1 Population numbers trapped at each trapping session

Trapping session	March 2005	June 2005	November 2005	June 2006	November 2006	March 2007
Captive	4	6	6	4	4	5
Wild	9	8	14	7	9	17
Total	**13**	**14**	**20**	**11**	**13**	**22**

2.1.1.1 Method used to trap captive population

Captive Gilbert's potoroos are captured using a shade cloth run and net on a fortnightly basis so that they can be regularly weighed and assessed. Biological samples for this study were collected from wild and captive potoroos during the routine handling sessions for management purposes in March, June and November in 2005, June and November 2006 and March 2007.

2.1.1.2 Method used to trap wild population

On Mount Gardner, Sheffield cage traps, wrapped in Hessian to minimize exposure to the elements, were set the afternoon prior to trapping and baited with a pistachio-nut flavoured muesli ball. These traps were checked at first light. When a potoroo was found it was transferred to a black cotton bag and a total weight obtained with the use of spring scales. The bag was also scanned with a Trovan® portable scanner (Trovan, North Ryde Sydney, Australia) for the presence of a microchip to enable identification of the individual animal. All potoroos were

assigned a number that correlated with their microchip number. If no microchip was found this typically indicated that the potoroo had not been caught previously, or was at an age unsuitable for microchipping (e.g. unfurred pouch young) when last caught. The potoroo was then briefly checked to determine its sex. If the individual was female, with a pouch young of a critical size (in terms of its vulnerability and survival) further disease sampling was not undertaken in order to prevent the possibility of pouch young loss associated with excessive handling. The potoroo was then released to the wild after undergoing the standard DEC morphometric measurements. The data and standard parameters recorded included the presence of pouch young, sex, head length, and weight. In pouch young, all the prior measurements as well as crown-rump length were recorded.

If the pouch young was not of a 'critical size' or had not been sampled previously in the week, the potoroo was returned to the closed trap in the black cotton bag while the rest of the trap line was checked. A faecal sample (usually four or five pellets) was collected from the trap floor or bag and then couriered overnight to Murdoch University for faecal flotation and parasitological examination.

The potoroos were transported from their trap sites in black cotton bags to the DEC utility truck on the main track lines. This walk was never more than 15 minutes. The bags containing the potoroos were then placed in a cardboard box on warmed heat pads whilst the animals awaited anaesthesia. Anaesthesia, for sample collection and physical examination, was performed on the tray compartment of a DEC vehicle in the field (Figure 2.2)

Figure 2.2 Anaesthesia and sample collection field set up on Mount Gardner and surrounds.

2.1.2 Anaesthetic protocol

To undertake the procedures required for disease testing the potoroo's nose was exteriorized from the black cotton bag and the animal was induced with Isoflurane (Veterinary Companies of Australia, Kings Park, NSW) delivered via face mask at 3.5% Isoflurane with an oxygen flow rate of 2.5L/minute. Loss of corneal reflex, withdrawal response as assessed via toe pinch, and general loss of muscle tone was closely monitored prior to unfolding the cotton bag and laying the potoroo in left lateral recumbency. Heart rate, and pulse characteristics were determined with the aid of a stethoscope, and respiratory rate was also recorded at this point. Five minute recordings of these parameters were made throughout the procedure unless circumstances or pathology warranted closer monitoring or intervention. Five minutes post induction most potoroos were able to be maintained on 1.5% Isoflurane and 2L/minute of oxygen. A pulse oximeter was available from June 2005 enabling the electronic monitoring of heart rate and oxygen saturation. These parameters were obtained by placing the probe on the external pinna or between the toes of the anaesthetised potoroo (Figure 2.3). Anaesthetic and general physical exam data sheets were completed every time an individual was anaesthetised for disease testing (Appendix 1). This

enabled anaesthetic and physical examination records of abnormalities to be retained for subsequent trapping sessions.

Figure 2.3 Anaesthetised Gilbert's potoroo maintained on face mask with pulse oximeter probe placed on external pinna.

After completion of the physical examination and sample collection the Isoflurane gaseous anaesthetic was turned off, the anaesthetic machine flushed to remove residual Isoflurane vapour and the potoroo was allowed to breathe oxygen at a 2L/minute flow rate. Ten milliliters (mL) of compound sodium lactate was delivered in the interscapular region subcutaneously, and the bladder was palpated and manually expressed. The total length of anaesthesia in a routine potoroo varied from ten to fifteen minutes.

The potoroo was maintained on oxygen for approximately five minutes until withdrawal responses returned, then placed back within its original black cotton bag and into a cardboard box warmed with heat pads for recovery. The potoroo was regularly checked and when sufficiently recovered to the point of ambulation and corresponding mental alertness it was released back to its original trap site.

2.1.3 Physical examination and biological sample collection protocols

Physical examination and biological sample collection were undertaken in a specific order to facilitate the sample collection process.

All four plantar aspects of the feet were swabbed with a moistened Amie's transport medium swab (Transwab®, Medical Wire & Equipment, England) to detect the presence of any environmental *Cryptococcus*. In males, the penis was then extruded and a comment made on the presence of any discharge and its characteristics. A dry wire swab (Mini-tip swabs, COPAN, USA) was then used to swab the prepuce for the detection of a *Treponema*-like organism via PCR assay. This swab was stored frozen at -20^{o}C degrees prior to transport via overnight courier to DAFWA. The prepuce was then swabbed with a moistened Amie's transport medium swab (Transwab®, Medical Wire & Equipment, England) which was first used to make a wet preparation smear for the detection of spirochaetes under dark field microscopy, then forwarded to the DAFWA for microbiological analysis and culture.

In females a similar methodology was used. The vagina was swabbed and a comment made on the presence of any discharge and its characteristics, and a dry wire swab (Mini-tip swabs®, COPAN, USA) was used for the detection of a *Treponema*-like organism via PCR assay. The vagina was then swabbed with a moistened Amie's transport medium swab (Transwab®, Medical Wire & Equipment, England) which was used to make a wet preparation smear for the detection of spirochaetes under dark field microscopy prior to being couriered overnight to DAFWA for microbiological analysis and culture.

A moistened Amie's transport medium swab (Transwab®, Medical Wire & Equipment, England) was collected from the cloaca for microbial culture including *Salmonella* testing, and couriered overnight to Murdoch University for culture and typing (if present).

An assessment of body condition and haircoat was then made. Body condition was graded from one to five, one being emaciated and five being obese. The potoroos were scored based on the amount of fat overlying their thoraco-lumbar spine and tail base. Optimal body condition was assigned to be between 2.5 and 3.5. The haircoat was examined for ectoparasites and any resulting skin inflammation. Microscopic ectoparasites were collected onto a slide and embedded in paraffin oil. Macroscopic parasites were placed into a plastic vial and sent to Murdoch University for identification.

A routine general physical examination was then performed. The abdomen was palpated, followed by palpation and manipulation of the musculoskeletal system. The left ear was examined using an otoscope (Welch Allyn, Australia) to check for the presence of ectoparasites, inflammation and patency of the tympanic membrane. The left eye was then checked using a direct ophthalmoscope (Welch Allyn) and a fundic examination was carried out.

A patch of hair overlying the left femoral triangle was then clipped with WAHL® clippers (Wahl Clipper Corporation and Unity Agencies, Victoria, 3180, Australia) and the skin prepared with a combined 50/50 chlorhexidine gluconate and 70% ethanol mixture. A 23G needle and a 3mL syringe were used to obtain a blood sample from the femoral vein, volume determined by body weight (P. Clark, personal communication February 20, 2005). The blood obtained was divided for different test procedures. A blood smear was made via the spreader slide technique for differential leukocyte counts and haemoparasite examination and couriered to Murdoch University. Approximately 0.5mL of blood was preserved in a 1.5mL ethylenedinitrilotetraacetic acid (EDTA) tube and sent to Murdoch University for a complete blood count. The remaining 3.5mL was placed into a plain-serum tube to be centrifuged later. Serum was removed and couriered overnight for biochemical analysis to Murdoch University, and subsequently forwarded to the Department of Primary Industries in Tasmania for *Toxoplasma* analysis via the Modified Agglutination Test (MAT) and the Direct Agglutination Test (DAT). The remaining serum was couriered to the University of Sydney for *Cryptococcus* infection testing via LCAT testing. If

excess serum was obtained this was frozen and stored for subsequent analysis if required. In the March 2007 trapping session 0.5mL whole blood was also collected for *Treponema* serology and sent to the Western Australian State Syphilis Research Laboratory in Nedlands WA, via PathWest Albany. The techniques for preputial biopsy will be discussed further in Chapter six. A summary of the samples submitted for testing is outlined in Appendix 2.

The potoroo was then rolled into right lateral recumbency. If an insufficient blood sample was obtained from the left femoral vein then venepuncture would be attempted on the right femoral vein. Routine physical examination of the haircoat, skin, ear and eye was then carried out as before.

The anaesthetic mask was then removed to facilitate assessment of the teeth and oral cavity. In particular tooth wear, cusp formation and any malocclusion was noted. A nasal swab was obtained with an Amie's culture medium moistened dry swab (Mini-tip swabs®, COPAN, USA) for culture and detection of *Cryptococcus*. Finally the bladder was palpated and manually expressed. This sample was then forwarded via overnight courier to Murdoch University for urinalysis, and sediment examination. Culture was indicated if large numbers of inflammatory cells were evident microscopically.

Following the completion of sample collection, anaesthesia was ceased and the potoroo was recovered as described above.

CHAPTER 3

The significance of *Cryptococcosis* on the health of the Gilbert's potoroo

3.1 INTRODUCTION

Cryptococcosis is caused predominantly by two species of basidiomycetous fungus: *Cryptococcus neoformans* (var. *grubii* and var. *neoformans*) and *Cryptococcus gattii* (formerly *C. neoformans* var. *gattii* or *C. bacillisporus*) (Malik *et al*. 2006). *C. neoformans* is commonly associated with bird guano, which provides a nitrogen rich environment favouring growth of the cryptococcal organism. Other environmental isolations have been made from rotting vegetables, wood, dairy products and soil (Sorrell and Ellis 1997). The environmental niche of *C. gattii* (molecular type VG1) has been associated with tree hollows, bark and plant debris of a number of eucalypt species, including *Eucalyptus camaldulensis* (river redgum), *E. tereticornis* (forest redgum), *E. grandis* (flooded gum) *E. rudis* and *E. gomphocephala* (Ellis and Pfeiffer 1996, Krockenberger, Malik and Canfield 2002, Malik *et al*. 1996, Malik *et al*. 2006). The most significant, consistent and widespread environmental association is with the tree hollows of *E. camaldulensis* (M.B. Krockenberger, personal communication, July, 2007). Human cryptococcosis caused by *C. gattii* (molecular type VGII) has a locally high prevalence in parts of the Northern Territory of Australia (140 cases per million compared with a nationwide prevalence of eight cases per million per year) (Chen *et al.* 2000) and has also been reported in Malaysia and Papua New Guinea (Sorrell and Ellis 1997). The natural distribution of the species of eucalypts known to be associated with the environmental niche of *C. gattii* does not extend to these areas, however in Arnhemland, in the Northern Territory of Australia, associations have been made with *E. miniata*, *E. tetradonta* (M.B. Krockenberger, personal communication, July, 2007). It is likely that environmental niches of VGII are still to be discovered.

Cryptococcosis is acquired from the environment, primarily via inhalation (Sorrell and Ellis 1997). Although rarely reported in marsupials other than the koala (Malik *et al.* 2006), cases of cryptococcosis do occur in Australian mammals, with cases in quokkas, wallabies and numbats found in the archives of the DAFWA (unpublished reports). Numerous marsupial cases are also found in the files of the Registry of Wildlife Pathology at Taronga Zoo (K. Rose. personal

communication, January, 2007). This suggests that cryptococcosis is not uncommon in marsupials.

In humans, disease caused by *C. neoformans* is classically associated with immunocompromised individuals, whereas *C. gattii* usually causes disease in immunocompetent hosts (Sorrell and Ellis 1997). In other mammals however, both *C. neoformans* and *C. gattii* can behave as primary pathogens of immunocompetent hosts (Malik *et al*. 2006). Common presentations in mammals include rhinosinusitis, pneumonia, and disseminated disease, including meningitis (Malik *et al*. 1996).

Cryptococcosis is not contagious or zoonotic. No reports of transmission exist from mammals to other animals or humans. One report ascribing zoonotic potential to this organism simply reports acquisition of cryptococcosis by an immunocompromised human from the faeces and soiled cage of an asymptomatic pet bird (Nosanchuk *et al*. 2000). Human to human transmission is also rarely reported and only under unusual circumstances i.e. a corneal transplant from an infected donor, (Husain, Wagner and Singh 2001). Only one case of vertical transmission has been reported of an HIV-positive mother with peripartum cryptococcal meningitis infecting her newborn (Cheng 2001)

Some features of the host-pathogen-environment interactions of cryptococcosis caused by *C. gattii* have been demonstrated in a naturally occurring model.

The koala has been studied for its capability to amplify the number of cryptococci in its environment. The koala feeds solely on the leaves of *Eucalyptus* spp. and therefore has a high environmental exposure to the disease (Sorrell and Ellis 1997). Koalas in areas with heavy environmental contamination with *C. n*. var *gattii* correspondingly have high incidences of nasal and skin colonization (Krockenberger, Malik and Canfield 2002). However, it was also shown that a high proportion of koalas can be heavily and persistently colonized by *C. n*. var. *gattii*, of

which only a small proportion will develop sub-clinical disease, and of these only a smaller proportion will progress to develop symptomatic cryptococcosis (Krockenberger, Malik and Canfield 2002). In the koala, tissue invasion causing cryptococcosis can be measured by serum cryptococcal antigen concentration (LCAT). Groups of koalas with a high incidence of nasal and skin colonization also have a high incidence of cryptococcal antigenaemia. However tissue invasion without the development of clinical signs is also commonly observed in koalas. As such, it has been reported that koalas with sub-clinical *Cryptococcus* infections will respond in one of the three following ways;

- The antigenaemia may negate over months owing to a favorable host immune response;
- The antigen titer may remain static, due to persistence of *C. gattii* most likely in the nasal cavity or lung; or
- The antigen titer may rise and clinical signs will then develop.

These are important findings and currently the only naturally occurring model of cryptococcosis.

3.1.1 Cryptococcosis in potoroos

Despite the high environmental prevalence of this fungus in eucalypt detritus (Ellis 1990), a foraging site for small potoroids, there are only two case studies of cryptococcosis in potoroids (Vaughan *et al*. 2007, Appendix 15). The absence of reported cases in potoroids could be a reflection of a small sample size, as there are few potoroos in captivity in Australia, where infections would most likely be noticed.

3.1.1.1 Cryptococcosis in a Gilbert's potoroo – case study

Cryptococcosis was diagnosed in a male Gilbert's potoroo which entered the captive breeding colony in January 1995. In March 1999 GP M7 had a brief sneezing and coughing episode and developed a hunched appearance. On clinical examination, it was trembling and appeared painful on palpation of its lower thoracic/lumbar spine. A tentative diagnosis of spinal muscle injury was

made but no treatment administered. The upper respiratory tract signs abated but the animal's appetite diminished and its body weight declined from 1096g to 947g (14%) in two weeks after which the problem seemed to resolve.

By February 2000, body weight had declined to 863g, a further 21% from initial, appetite had diminished and the animal presented with another episode of apparent discomfort with a hunched appearance. Haematological and plasma biochemical testing revealed leukopenia and the presence of intra-erythrocytic *Theileria*-like organisms. Similar organisms had been seen in the blood of 100% of the potoroos in the captive colony without associated clinical signs (D.Forshaw, personal communication, February 10, 2006). No treatment was given and the animal improved over a week but was seen on one occasion to be 'circling' its pen.

Further significant weight loss occurred over the next six months. In August 2000 at a body weight of 748g, (32% below initial weight) the potoroo appeared sluggish and ataxic. It had pale mucous membranes, was estimated to be 5% dehydrated (based on skin turgor) and was hypothermic (rectal temp 32.8º C). It was treated with 50mL of warmed subcutaneous fluids (2.5% glucose in 0.9% saline) once daily for three days but then deteriorated, displayed forelimb paresis, and was seen to intermittently circle and stumble to the left. No withdrawal reflex could be elicited in the left foreleg with noxious stimuli and marked atrophy of the left scapular muscles was present.

Supportive therapy with the anabolic steroid norandrosterone laurate (1mg) and Vitamin B and E as well as 50mL once daily of the aforementioned subcutaneous fluid therapy was administered. To address the possibility of bacterial meningitis, the animal was treated with the broad spectrum antibiotic amoxicillin/clavulanic acid (12.5mg) subcutaneously (SC) daily for three days. Treatment was continued for five days without clinical improvement. Despite several attempts at venepuncture, blood collection for haematology and biochemistry was not achieved until the potoroo was anaesthetised by gaseous induction anaesthesia (5% isoflurane, and 2L/min

oxygen). Whole body radiographs were also taken at this time. The only radiographic abnormality noted was mild bilateral lucency of the nasal cavity. Haematology and plasma biochemical analyses were normal apart from a raised creatine kinase activity (4991 U/L) attributed to prolonged recumbency. Serum agglutination tests for *Toxoplasma* IgG and IgM antibodies were negative. Urine was collected and urine dipstick analysis showed 2+ glucose and 1+ protein.

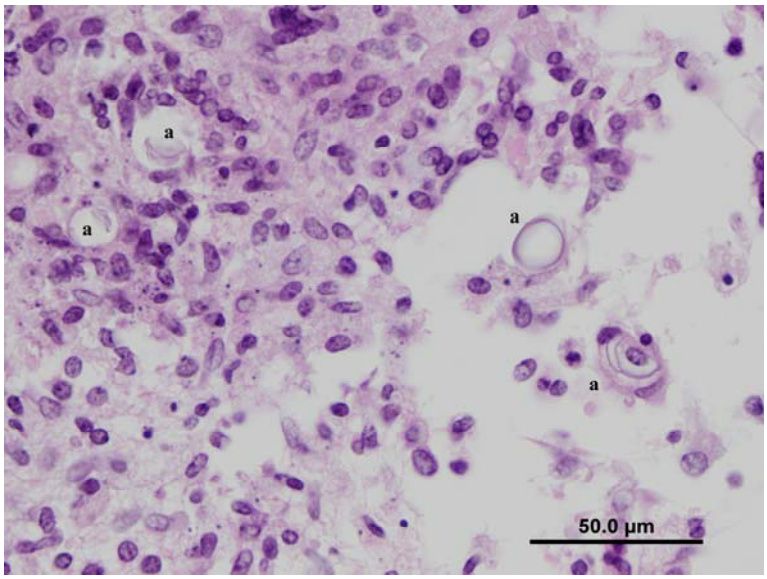
The potoroo recovered from anaesthesia to the point of ambulation but an hour later was found comatose and twitching. At this stage horizontal nystagmus was noted. Diazepam 0.1 mg intra-muscular (IM) initially seemed to calm the potoroo but later convulsions ensued. Further diazepam (0.2 mg IM) was administered as an anticonvulsant as was dexamethasone (0.1 mg IM). At this time, marked anisocoria and lateral nystagmus were observed. The potoroo settled but began convulsing again and for the next 12 hours the patient was treated regularly with diazepam to control convulsions. Finally it was anaesthetised again and blood collected before it was euthanased with intracardiac pentobarbitone. The LCAT was negative (Malik *et al.* 1996) using a Cryptococcus Antigen Latex Agglutination System (CALAS) kit incorporating a pronase pretreatment step (Meridian Bioscience). This test detects soluble cryptococcal capsular polysaccharide antigen in body fluid samples including serum, urine or cerebrospinal fluid (CSF). The pronase pre-treatment step increases sensitivity (Connolly *et al.* 1999).

Necropsy revealed extensive fat reserves, despite the 32% loss in body weight. Stomach contents were stained with blood and there was diffuse haemorrhage from the cardia. There were multifocal irregular areas of congestion with central focal yellow areas up to 3mm in diameter throughout the liver. In the subcutaneous tissues of the hind legs there was patchy congestion and haemorrhage, particularly in the musculature of the pelvis and proximal hind limb around the sciatic nerve. No gross lesions were noted in the central nervous system. No other significant abnormalities were detected at necropsy.

On histopathology, the most significant findings were in the CNS with a severe multi-focal granulomatous meningo-encephalomyelitis. Within vacuoles in macrophages there were intra cellular yeast-like bodies with surrounding capsule-like spaces (Figure 3.1). No obvious budding was identified. Lesions were widespread in the meninges of the cerebral cortex and in the sub-ependymal areas of the lateral ventricles. Particularly prominent inflammatory foci were seen in the roof of the fourth ventricle and around one vestibular nerve. A similar lesion was evident in the spinal cord at the level of brachial plexus. In the white matter of the cord in this region there was a patchy vacuolar change with occasional swollen axons. In the cerebellum, numerous Purkinje cells were acutely necrotic. There was also multi-focal moderate acute hepatic necrosis, superficial localised moderate gastric mucosal necrosis, diffuse acute mild renal tubular necrosis, multi-focal mild membranous glomerulonephritis and mild multi-focal acute rhinitis. Immunohistochemistry, utilizing a panel of antibodies directed against different serotypes of the *Cryptococcus* species complex (Krockenberger *et al.* 2001*)* was performed*,* labeling the organisms in a manner consistent with a diagnosis of *Cryptococcus neoformans* as the causative organism.

Although the exact source of the organism was undetermined in the Gilbert's potoroo, contamination of the enclosure by Bronzewing pigeon faeces was considered a possibility, given the environmental associations of *C. neoformans* with weathered bird guano. To address this, preventative measures were carried out which included removal of the top soil and spelling of the enclosure for a year.

Figure 3.1 *Cryptococcus neoformans* in a Gilbert's potoroo. Granulomatous inflammatory lesion in the spinal cord with large distorted yeast forms (a). 400X Haematoxylin and Eosin.



3.1.1.2 Cryptococcosis in a long-nosed potoroo – case study

In November 2004, a 4.5 year old female long-nosed potoroo from the Perth Zoo colony presented with a loss of 80g, 27% of its body weight over a six week period. This potoroo had a pouch young and a young at foot, both of which were removed to minimise further nutritional strain from lactation and to rule out competition for food with the young at foot.

In addition to weight loss, early presenting signs included hind limb weakness, gluteal muscle atrophy, and a rough hair coat. Physical examination under isoflurane anaesthesia, including dental examination and whole body radiographs, did not detect further abnormalities. Haematology and serum biochemistry were normal, except for a mild hyperbilirubinemia. *Toxoplasma* serology was negative, and no evidence of intestinal parasitism was detected on faecal flotation. There was no clinical response to empiric therapy with combined amoxycillin-clavulanic acid 12.5mg SC and 25mL of SC fluids, both administered daily for five days.

The potoroos condition deteriorated over the subsequent five days at which point it was again anaesthetised for the administration of IV fluids, and parenteral vitamins (B and E) and venepuncture performed for repeat haematology and serum biochemistry. No significant findings

were detected. Therapy under general anaesthesia was repeated the following day, and antibiotic therapy was changed to enrofloxacin (5mg SC s.i.d). Meloxicam (0.2mg SC s.i.d) and norandrostenolone laurate (1mg SC) were also administered. An indwelling IV catheter was placed in the lateral tail vein.

Seven days following initial anaesthesia, a slight and progressive head tilt to the right, circling to the right and prolonged recumbency became apparent. Bilateral exopthalmos, visual deficits, and progressive ataxia then ensued. The pupils became fixed and dilated, and upon fundic examination, retinal haemorrhage and changes consistent with optic neuritis were evident. Blood was taken for serum cryptococcal antigen testing (LCAT) (Malik *et al*. 1996) using a CALAS kit (Meridian Bioscience) incorporating a pronase pretreatment. Presumptive treatment for cryptococcosis was initiated with itraconazole*,* 20mg/kg s.i.d *per os* (PO). The ocular lesions were treated with 0.5% dexamethasone topical drops q.i.d. Enrofloxacin was continued prophylactically in case of secondary bacterial infection.

The LCAT test result was positive with a titer of 1:32. While the titer of the positive test was not spectacularly high, it was consistent with tissue invasion by the fungus, and in combination with the clinical signs was consistent with a diagnosis of neural and ocular cryptococcosis (Malik *et al*. 1996).

Inappetence continued, necessitating supplemental syringe feeding. Although the potoroo appeared brighter in demeanor following the start of antifungal treatment, circling to the right persisted and was exacerbated by stress. Ocular lesions showed no improvement. After one week of treatment with itraconazole, anti-fungal therapy was changed to fluconazole (10mg/kg PO b.i.d). Additionally 0.7mg of amphotericin B (0.14mL of the 5mg/mL Fungizone ^{R} solution) was administered subcutaneously twice weekly, diluted in 50ml of 0.45% NaCl and 2.5% glucose. (Figure 3.2). The Amphotericin B infusion was administered under anaesthesia to minimise

stress. No cryptococcal organisms were cultured from nasal flushes, and repeat radiographs did not show any evidence of pulmonary involvement.

Figure 3.2 The SC administration of dilute amphotericin B given under light gaseous anaesthesia to minimise stress in a long-nosed potoroo with suspected cryptococcosis.

Ophthalmoscopic examination 25 days following the initial anaesthesia revealed persistence of fundic changes consistent with a significant optic neuritis and absence of the pupillary light reflex in the right eye (Figure 3.3). Similar but less severe changes were present in the left eye. These findings combined with the potoroos behaviour were consistent with partial blindness. The ocular dexamethasone drops were reduced to b.i.d to minimise handling. Repeat monitoring of haematology and serum biochemistry indicated normal renal function, despite the potentially nephrotoxic effects of amphotericin B.

A repeat LCAT, 40 days after the initial positive LCAT, showed a greatly increased titer of 1:8192. Usually a decline in titer lags behind clinical improvement (Connolly *et al.* 1999), yet this was not apparent. Consequently, a decision was made to increase doses of amphotericin B to 0.9mg per treatment and to increase the fluconazole to 20mg PO b.i.d.

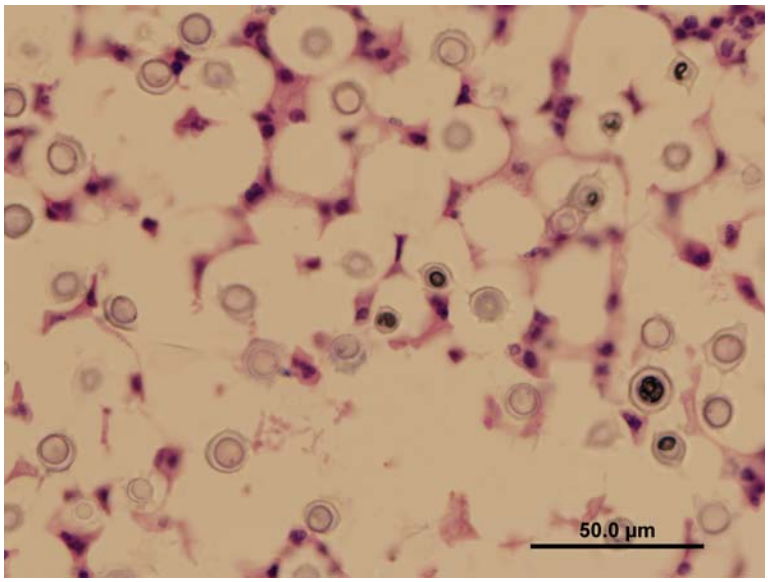
Figure 3.3 Exopthalmos and fixed dilated pupils were prominent.

One week later, polydipsia became evident, however, there was no evidence of azotaemia. Two weeks following implementation of the new dosage regime, the animal became inappetant. Conjunctivitis developed in the left eye, and was treated with chloramphenicol ointment b.i.d. Given the lack of improvement in neurological status, and the onset of secondary problems, a decision was made to euthanase the potoroo. The animal was anaesthetised, then euthanased with IV pentobarbitone.

Histopathology revealed disseminated meningoencephalitis, optic neuritis, granulomatous rhinitis, and pneumonia. In both the respiratory and nervous tissue, there were multi-focal lesions of a typical “soap-bubble” appearance comprised of large numbers of narrow-necked budding yeasts and large spaces surrounding them corresponding to fungal capsular material and fixation

artefact. (Figure 3.4). *Cryptococcus gattii* was cultured from the cerebral tissue, and from a sample of cerebrospinal fluid collected aseptically immediately post mortem.

Figure 3.4 *Cryptococcus gattii* in a long-nosed potoroo. Typical 'soap-bubble' lesion in olfactory lobe of the cerebral cortex. 400X Haematoxylin and Eosin.



Definitive identification of the source of infection in the long-nosed potoroos was not found although the practice of recycling *Eucalyptus* sp. as browse at the Perth Zoo is a strong possibility. Recycled koala browse was being offered to the long-nosed potoroos at the time of infection and represents a potential source of transmission. This practice has since ceased.

3.1.1.3 Discussion of cryptococcosis in potoroos

Some common features are evident in these two cases even though two different species of *Cryptococcus* were involved and different species of potoroo were affected. Severe granulomatous meningoencephalitis was considered to be the cause of the clinical syndrome in both potoroos with lesions in other organs considered secondary. Hind limb weakness, muscle atrophy, ataxia and circling were consistent clinical findings. Visual deficits were evident in both and marked retinal haemorrhage and optic neuritis were seen in the long-nosed potoroo.

Clinical diagnosis of cryptococcosis was confirmed in the long-nosed potoroo by the positive LCAT test and the clinical experience gained from handling the case in the Gilbert's potoroo.

It is likely in both cases that the respiratory tract was the primary site of disease following inhalation of infectious propagules into the lung with subsequent central nervous and haematogenous dissemination. Alternatively, the episode of respiratory disease in the Gilbert's potoroo could possibly have been caused by a primary cryptococcal rhinitis. However, the lack of lesions in the nasal cavity and the long period of time between this episode and the illness preceding death makes this unlikely.

3.1.2 Treatment

Although treatment regimes are yet to be established for cryptococcosis in marsupial species there is sufficient experience in the treatment of cryptococcosis in cats and dogs to extrapolate with some degree of confidence.

Amphotericin B (often combined with 5-flucytosine) is recognized as the gold standard in the treatment of cryptococcosis, owing to the weight of evidence-based medicine supporting its use and because it is fungicidal rather than fungistatic (Legendre and Toal 1995). However, given its potential for irreversible nephrotoxicity, it is usually reserved for life-threatening cases with CNS involvement and cases unresponsive to azoles. The decision was made to initially commence treatment with itraconazole, an agent that could be given orally without recourse to general anaesthesia. However, it soon became clear that itraconazole was having little effect in reversing neurological signs, and therapy was therefore changed to the combination of amphotericin B and fluconazole. Fluconazole achieves better CNS penetration than itraconazole, with generally fewer side effects (Legendre and Toal 1995). Although the use of 5-flucytosine may have been helpful, it was not possible to obtain this agent in a timely fashion or in a suitable formulation.

Historically, amphotericin B was only administered IV to human, canine and feline patients. However, newer subcutaneous infusion methods (Malik *et al*. 1996) not only slows absorption of the potentially nephrotoxic drug, and promotes diuresis to help minimise nephrotoxicity, but also makes administration possible under manual restraint. Professor Richard Malik and colleagues (R. Malik, personal communication, June, 2006) have used this approach in a variety of species including koalas and a cheetah. Although the potoroo still required light general anaesthesia for administration of amphotericin B, its small size and lack of robust veins for venepuncture made the SC route a practical route of administration.

In cases of optic neuritis, many authorities recommend concurrent administration of corticosteroids. However, owing to the underlying infectious aetiology, steroids were initially withheld in favour of non-steroidal anti-inflammatory drug (NSAID) therapy. Corticosteroids were however introduced following the second administration of amphotericin B, when neurological signs became worse immediately following treatment. This phenomenon was thought to be related to the inflammatory effects of the fungicidal activity of amphotericin and potential osmotic activity of fungal capsular polysaccharide released from dead and dying organisms in the CNS (Malik *et al.* 1996). In an attempt to pre-empt potential adverse neurological sequelae, premedication with dexamethasone (0.5mg/kg) before subsequent amphotericin B infusions was performed.

Despite therapeutic failure in these cases, the treatment techniques outlined above should be instrumental in formulating any treatment plan should any further cases of cryptococcosis be identified in potoroo species. Earlier diagnosis and initiation of treatment are likely the most important aids to survivability.

3.2 MATERIALS AND METHODS

The Gilbert's potoroos were anaesthetised as previously described. At the start of the procedure a wet Amie's transport medium swab was taken from the plantar surface of all feet, being vigorously rubbed on the surface of the extremities and digits until the swab became blackened with dirt and debris. This site was chosen as it has been found that koalas often harbour the organism in the web spaces of their claws (Krockenberger *et al*. 2002). A nasal swab was then taken using a urethral dry swab (Copan-USA), this was chosen owing to the small diameter of the nares in this species and then placed into Amie's transport medium. Blood was collected from the femoral vein and placed into a serum tube which was later centrifuged and a 0.5ml serum sample was sent to Dr Mark Krockenberger at the University of Sydney on ice via overnight courier for LCAT analysis. The nasal and feet swabs were also sent to Dr Krockenberger on ice for culture.

In June 2005, environmental swabbing was undertaken of the captive Gilbert's potoroos enclosures. Four wet Amie's transport medium swabs were taken from each pen. These were vigorously rubbed on bark, detritus and trees within the enclosure for cryptococcal culture. Environmental swabbing was undertaken as high concentrations of the organism (up to 1000 cfu/g) are found in woody debris in the hollows of aged trees which being moist and lacking in sunlight provide a preferable environment for cryptococcal growth. (Sorrell and Ellis 1997). These samples were also sent on ice to Dr Mark Krockenberger at the University of Sydney via overnight courier for culture.

3.3 RESULTS

One aged female GP F86 within the captive colony was found to have five colonies of Cryptococcus on a nasal swab in June 2005. This individual was captured from the wild in February 2005 and was placed into quarantine at the captive Gilbert's potoroos pens pending

translocation to Bald Island. Prior to translocation, potoroos are preventatively treated with the macrocylic lactone, Ivermectin using the standard 200µg/kg dose rate and sprayed with pyrethrin, N-Octyl-bicycloheptene and Piperonyl butoxide (Fido's fre-itch concentrate®, Mavlab,Queensland) a flea, lice and tick adulticide concentrate, diluted 2ml to 200ml water. Four days following treatment GP F66 was translocated to Bald Island for three weeks. Upon return disease screening as described in the material and methods section was undertaken. Five colonies of *Cryptococcus* were found on nasal swab. Serological evidence of tissue invasion via LCAT titer was negative. No cryptococcal organisms were cultured from environmental soil samples (from the captive enclosures) or feet swabs. No overt clinical signs including evidence of respiratory compromise from nasal colonisation or pneumonia were present. There was no evidence of ocular signs including optic neuritis or neurological dysfunction. This female did however have biochemical and urinalysis values consistent with chronic renal disease. A follow up nasal swab sample one month later was negative on culture.

This individual continued to be housed in the captive colony upon return from Bald Island and was found dead on the 23rd of December 2005. Post mortem examination revealed the cause of death to be a parasitic hepatitis. Throughout the liver multiple white thick walled cystic structures up to 1.5cm were seen. On cross section some contained coiled worms identified as *Ophidascaris*. Other cysts contained a purulent material. On one edge of the liver a worm was seen protruding from the liver and focal haemorrhage was seen around the exit point and worm. The kidneys were slightly enlarged and pale, with the left kidney containing multiple cysts (up to 3mm in diameter) throughout the cortex. Given the history of familial renal oxalate nephrosis in this species, and evidence of renal tubular degeneration a probable familial renal oxalate nephrosis was presumed. The apparent cause of death however was acute haemorrhage into the abdomen associated with the emergence of the worm from the liver. There was no evidence of cryptococcosis on histopathological examination.

No other wild or captive potoroos showed any evidence of cryptococcosis based on clinical signs, nasal and feet swabs for culture nor any serological evidence of antigenaemia or tissue invasion via LCAT titers over the study period.

3.4 DISCUSSION

The habitat of potoroos should be considered as a factor that could increase susceptibility to cryptococcosis. Potoroos typically rest in detritus (which may contain guano) and on moist leaf litter substrates. Given the known environmental associations of *C. gattii* and *C. neoformans* this may expose the potoroos to large concentrations of infectious propagules, increasing their potential for exposure and subsequent disease. Studies in captive koalas have made a substantial link between the level of environmental presence of *C. gattii* and the prevalence of sub-clinical and clinical cryptococcosis (Krockenberger *et al.* 2002).

Female GP 66 only had a low level of nasal colonization and no circulating antigenaemia. It is most likely that she had a low level nasal colonization which did not progress to tissue invasion due to effective innate and possibly adaptive surface immunity. This was supported by the repeat negative nasal swabs. Non-clinically affected koalas, cats and dogs often have colonization of mucosal membranes (including nasal passages) without tissue invasion (Connolly *et al.* 1999, Krockenberger *et al.* 2002). As such, a positive nasal culture should be treated with caution if supportive evidence of inflammation or other evidence of invasion is lacking.

Given the lack of positive LCAT serology throughout the study it remains unknown if potoroos like the koala, exhibit similar tendencies in regards to surface mucosal colonization compared to tissue invasion. Whereby tissue invasion (antigenaemia), without the development of clinical signs may occur. Limited tissue invasion in asymptomatic koalas with low positive antigen titers has been confirmed (Krockenberger, Canfield and Malik 2002). However, in rat models cryptococcal polysaccharide antigens do not passively cross the lining of the respiratory tract to

enter the blood stream (Krockenberger, Canfield and Malik 2002). As such, a positive serum antigen titer implies tissue invasion by the organism. The Perth Zoo long-nosed potoroo had developed clinical signs prior to LCAT diagnostic serology being undertaken and the Gilbert's potoroo, GP M7 showed a negative LCAT despite advanced stages of disease. As such further studies are indicated to determine the pathogenicity of antigenaemia if no corresponding clinical signs are evident. Follow up LCAT serology in light of such findings in conjunction with nasal swabbing would be indicated as would regular monitoring for the onset of clinical disease.

Although it is possible that both species of *Cryptococcus* can cause disease in immunocompetent potoroos, it is worth considering the prior immune status of animals with cryptococcosis. A study of mycobacterial disease in long-nosed potoroos suggests that susceptibility to this disease is related to immunological status and/or regulation (Young, Holz and Deane 2002). The infection of the Gilbert's potoroo GP M7 with *C. neoformans*, typically reported in immunocompromised individuals is of interest in regards to this individual being removed from the wild as a pouch young with his mother GP F4 and being brought into captivity. The subsequent changes in homeostasis following movement into the captive environment are of potential concern. However, it is unknown whether or not this individual was immunosuppressed at this time or if any immunosuppression occurred subsequently as no specific immune function testing was undertaken. GP M7 was brought into captivity from Hakea Gully in December 1994, and started to show obvious weight loss from February 2000, with neurological deficits observed in late August 2000. Euthanasia was conducted on the 1st of September 2000. The six year time lapse from entering the captive colony to the development of clinical signs may suggest that this individual became subsequently immuocompromised. As this individual did not display any signs of ill health or disease in the six years preceding its death, as would be expected from an immunocompromised individual.

Wild caught GP F66 was brought into the captive colony and then translocated to Bald Island. A case could certainly be constructed that the changes in environment and husbandry would lead to

a disruption in homeostasis. Yet, the subsequent clearing of infection suggests that this individual was able to mount an effective immune response against the organism as she did not become systemically infected.

In species with small population sizes the effects of disease can be catastrophic. Over time, these small populations can also lose genetic variability as a result of processes including genetic drift and/or inbreeding, which may in turn increase susceptibility to disease (Hutchins, Foose and Seal 1991). The Gilbert's potoroo, with its low levels of genetic variation certainly fits into this category (Sinclair 2001). Constraints in blood volume collection owing to body size and other disease testing requirements as well as logistical constraints with the diagnostic immune function testing laboratory being situated at Macquarie University in Sydney and shipping time, meant immune function testing via mitogen (PHA) driven proliferation assays (Ashman *et al.* 1976, Brozek *et al.* 1992, Buddle *et al.* 1992, Wilkinson *et al.* 1992) was not able to be conducted. Immune function testing of this species would certainly be warranted if the above constraints were no longer an issue. This would shed light on whether the Gilbert's potoroos could be more prone to the development of disease such as *C. neoformans* typically seen in immunosuppressed individuals.

Prevention of environmental exposure to cryptococcal organisms is difficult, as *C. neoformans* is ubiquitous in the environment. Avoidance and/or environmental control of bird guano may help prevent disease due to *C. neoformans* var. *neoformans*. After the death of GP M7 the soil in the enclosure was removed and the enclosure was spelled for one year. Pigeon droppings may remain infectious for up to two years (OIE 2005) however given that all access to bird guano had been removed, the year long spell following soil removal was probably not indicated. Other recommendations prior to the removal of the soil include chemical decontamination with 1% sodium hypochlorite, or iodine (OIE 2005). Other disinfectants, including glutaraldehyde, phenolic disinfectants and formaldehyde, pose risks to both the operator and the potoroos in the adjacent pens and as such would not be recommended. If these are not available, simply wetting

down the soil prior to removal will reportedly help to prevent aerosolisation. In cases of *C. gattii* infection, avoidance of *Eucalyptus* trees may reduce the risk of exposure.

It is difficult to ascribe significance to the one case of cryptococcosis caused by *C. neoformans* given the small population size of the Gilbert's potoroo, but given that only one case has been seen in twelve years, and that no serological evidence of antigenaemia has been seen over the study period its current prevalence within the population seems minimal. This may be a reflection of the altered management practices to minimize access to bird guano following diagnosis in GP M7.

3.5 CONCLUSION

Cryptococcosis represents a potential threat to the survival of both the critically endangered Gilbert's potoroo and the vulnerable long-nosed potoroo. Cryptococcal screening via nasal swab, LCAT titers and environmental sampling should be considered in captive breeding and translocation health screening protocols for potoroos. Preventative measures including minimising access to bird guano, i.e. by providing closed roof pens, should be implemented as well as quarterly sand changes of pens. The use of *Eucalyptus* spp. should be minimized and no recycling of *Eucalyptus* sp. used as prior koala browse should be undertaken.

Potoroos exhibiting inappetance, weight loss, hind limb weakness and neurological deficits including ataxia and circling, plus or minus altered vision should be screened for cryptococcosis via LCAT and nasal swab. If the patient is intractable and anaesthesia is required (as will most likely be the case) a screening thoracic radiograph should also be undertaken, and if facilities allow a CT or MRI scan.

Should a further clinical case of cryptococcosis occur, the treatment regime trialed in the long-nosed potoroo should be instituted in light of assessment of the relative stress of treatment

versus clinical staging of the disease. A wild individual with advanced stages of disease may not be a candidate for treatment given the intensiveness of handling required, where as a member of the captive colony would be a more amenable candidate.

CHAPTER 4

The significance of parasites on the health of Gilbert's potoroo

4.1 ECTOPARASITES

4.1.1 Introduction

Current knowledge about parasites and parasitism in Australian native mammals is continually evolving. The life-cycles of few are known, however as the majority of parasites belong to taxonomic groups related to those affecting domestic species, the basics of these unknown life cycles can generally be inferred from those of more familiar parasites (Dunsmore 1976).

Macropods can be host to a number of ectoparasites including ticks, fleas, lice, and mites (Blyde 1999). The general clinical signs resulting from these infestations have included dermatitis, alopecia, crusting, erythema and pruritus (Blyde 1999). Often the most sparsely haired regions are affected including the pinna, axilla, commissures of the mouth, and the abdominal, scrotal and inguinal areas. Anecdotally, wild Gilbert's potoroos have a long history of parasitism with ticks, lice and mites, however in the past identification to species level was not conducted. To definitively diagnose and identify infection, a skin scraping or skin biopsy showing the presence of ectoparasites is required (O.Callaghan *et al.* 1994). *Sarcoptes* is the exception, as this mite is notoriously difficult to identify. Multiple skin scrapings on multiple occasions are required (M. Burrows personal communication, June, 2005).

Ectoparasite treatment in macropods usually involves Ivomec® (Ivermectin) topically administered at 200µg/kg weekly for four to six treatments or Cydectin® (Moxidectin) 200-500 µg/kg orally, topical or parenterally (Blyde 1999). Captive Gilbert's potoroos are sprayed with pyrethrin, N-Octyl-bicycloheptene and Piperonyl butoxide (Fido's fre-itch concentrate®, Mavlab, Queensland) a flea, lice and tick adulticide concentrate, diluted 2mL to 200mL water. They are also administered the macrocyclic lactone Ivomec® (Ivermectin)

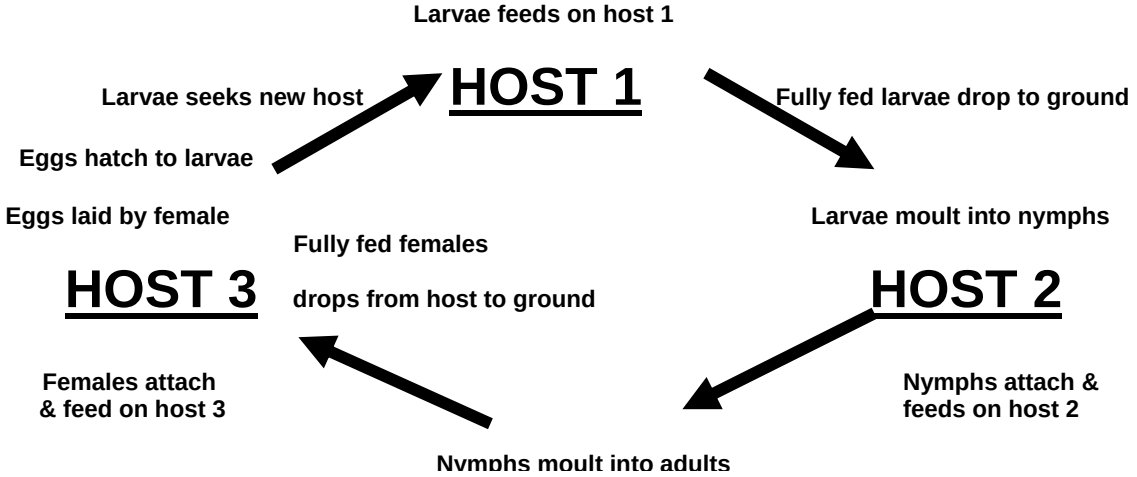
(Merial, Australia) topically at 200µg/kg upon entering the captive colony and routinely throughout the year.

4.1.1.1 Ticks

Approximately 70 species of tick are present in Australia (Buckett and DeMarco 1997). In domestic animals such as cattle, attachment to the host can cause skin irritation and subsequent ulceration and secondary infection. Myiasis can also develop causing annoyance and restlessness, often leading to a loss of weight and condition (Kaufmann 1996). Heavy infestations have been associated with anaemia, as adult female ticks can engorge up to 10mL of blood (Kaufmann 1996). Ticks have been commonly reported in most wild macropod species (Speare *et al.* 1989). Pathology has been associated with *Amblyomna triguttatum* and *Haemaphysalis bancrofti* (Nuttall and Warburton 1911) where mild focal inflammation was found at the site of attachment. Ticks can be divided into two families, hard (Ixodidae family) or soft (Argasidae family).

The majority of ticks reported from Australian marsupials belong to the *Ixodes* genus. *Ixodes* ticks are brown and eyeless (Speare *et al.* 1989). There are three parasitic stages in their life cycle; larval, nymph and adult forms. (Figure 4.1). Dependent on the individual species, ticks have either one, two or three hosts (Roberts 1970). Most Australian ticks are three host ticks, with the exception of *Boophilus microplus*, a one host tick (Roberts 1970).

Figure 4.1 Life cycle of a three host tick



Of special note is the paralysis tick *Ixodes holocyclus.* This tick is found mainly along the coast of eastern Australia. Tick paralysis in humans is associated with an ascending, afebrile, symmetrical paralysis, following a caudal to cranial progression. The symptoms may include increased weakness of the limbs, rashes, headache, flu-like symptoms and partial facial paralysis (Kauffman 1996).

Most domestic animals appear susceptible to tick paralysis, which can be fatal in the young. Concern has been raised for native marsupials entering tick endemic areas as the toxin may be lethal to those not previously exposed and may result in severe associated dermatitis (Beveridge 1993). The paralysis tick is commonly found throughout the pelage of bandicoots and brushtail possums, in tick endemic areas. Anecdotal reports have described an associated tick-like paralysis in the brushtail possum (Speare *et al.* 1989). In these cases paralysis was relieved with the removal of ticks.

Ornithodoros gurneyi, known also as the kangaroo tick is usually found in inland Australia in dry scrubland soil (Speare *et al.* 1989). In humans the bite causes local inflammation and irritation and can cause mild systemic effects including malaise. One report exists of a wild kangaroo developing neurological signs following infestation with *Ornithodoros gurneyi*. Flight reaction diminished, ataxia was observed and recovery took 15 minutes following removal of the ticks (Henry 1938). The possibility of other concomitant agents causing neurological deficits should also have been considered in this case.

Many other ticks have been reported to parasitise non-eutherian mammals (marsupials and monotremes) including *Ixodes ornithorhynchi,* which is seen in the platypus in large numbers without associated effect (Roberts 1970); the wombat tick, *Aponomma auruginans*, seen in large numbers in the common wombat (*Vombatus ursinus*) (Beveridge 1993); *Haemaphysalis bancrofti* and *Amblyomma triguttatum* in northern Australia in large macropods (Beveridge 1993); and *Ixodes tasmani*, the common marsupial tick (Neumann

1899), occurring in southern Australia with a host range including many families of marsupials, monotremes and rodents.

The long-nosed potoroo has been reported to be infected by *Ixodes australiensis* (Neumann 1964) in Tasmania. This *Ixodes* species has also been reported in Western Australia at Dryandra, Kojonup, Cranbrook, Northcliffe, Bald Island, Karragullen, Nornalup and Albany (Roberts 1964). Hosts include the quokka, brush-tailed bettong, burrowing bettong (*Bettongia lesuer*), cattle (*Bos taurus*) and humans.

4.1.1.2 Fleas

The geographic range of known flea hosts is constantly expanding, many flea species have now been reported to parasitise marsupials (Dunnett 1974, Oakwood and Spratt 2000). Fleas identified tend to be those from the most heavily studied species used for research purposes, including endangered species, and road kill surveys which primarily involve dasyurids and macropods.

Fleas, from the order Siphonaptera family Pulicidae, including *Echidnophaga aranka* and *Xenopsylla vexabilis*, have been observed in low numbers on the northern quoll (*Dasyurus hallucatus)*. *Echidnophaga aranka* has also been reported to infest the burrowing bettong in Western Australia (Dunnett and Mardon 1974). Similarly, *Echidnophaga ambulans* only known previously to infect short-beaked echidnas (*Tachyglossus aculeatus),* from south-eastern and south-western Australia has reportedly infected the northern quoll (Dunnett and Mardon 1974). Although this record represents a large extension of the known geographic range of *Echidnophaga ambulans*, it is uncertain whether these fleas were naturally associated with northern quolls or short-beaked echidnas, which were also observed in the area.

4.1.1.3 Lice

Most wild macropod species carry biting lice of the family *Boopidae* (Keler 1971). No associated pathogenic changes have been reported (Speare *et al.* 1989). However, heavy infestations have been correlated with poor health status and reproductive activities in marsupial species. *Heterodoxus* spp. was reported in very large numbers in three bridled nail-tail wallabies (*Onychogalea fraenata*) (Turni and Smales 2001) however these heavy infestations were only seen in old and sick individuals (Seddon 1967, Nelson 1984). In this study, high louse numbers were correlated to poor health status. Heavy infestations with the louse, *Boopia uncinata,* were observed on the back and rump of male northern quolls during the mating and die-off periods (Oakwood and Spratt 2000). Lice were recorded on only one female and this was a light infestation, observed a few days post-mating. When trapped three weeks later, no lice were found (Oakwood and Spratt 2000) This disappearance was thought to be a natural occurrence rather than an artifact of collection, as only a small proportion of lice were collected from any females on any occasion.

4.1.1.4 Mites

Domrow (1987) reported that 13 species of mesostigmatic mites parasitise macropods. Of these, the trombiculid mite has generated the most literature regarding host response. The adult and nymph stage of trombiculid mites are soil living while the larval stage parasitise vertebrates. They tend to exhibit a low level of host specificity and are found on numerous species including rodents, bandicoots and other marsupials (Domrow 1987). They have generated medical interest owing to their vector status in the transmission of scrub typhus, *Orientia tsutsugamushi* (Beveridge and Spratt 2003). In northern Queensland, Speare *et al*. (1983) reported the presence of large numbers of trombiculid mites on the skin of the medial thighs of agile wallabies *(Macropus agilis)* and found associated skin inflammation up to 5mm from the attachment site. Often there was a central depression containing encrustations. Oakwood and Spratt (2000) reported similar orange-coloured larval trombiculid mites, *Guntheria coorongensis,* on the ear base, and the rim of the pouch in the

northern quoll in the tropical savanna of the Northern Territory. They were found to be most abundant in the late wet season and early dry season.

4.1.1.5 Rickettsiae

Rickettsiales are obligate-intracellular Gram-negative coccobacilli that parasitise eukaryotic cells (Biberstein and Zee 1990). They have been included in this chapter as they are parasites of arthropods (lice, fleas, mites, ticks and insects). Some of the rickettsiales can also be passed transovarially in ticks and mites (Biberstein and Zee 1990).

Rickettsia rickettsii, the agent of Rocky Mountain Spotted Fever, is the best known of the rickettsiales (Biberstein and Zee 1990). The organism that causes Rocky Mountain spotted fever is transmitted by the bite of an infected tick. The American dog tick (*Dermacentor variabilis*) and Rocky Mountain wood tick (*Dermacentor andersoni*) are the primary athropods (vectors) which transmit the disease in the US (CDC 2008). The brown dog tick (*Rhipicephalus sanguineus)* has also been implicated as a vector as well as the tick *Amblyomma cajennense* in countries south of the US. *Rickettsia rickettsii*, is the most severe tick-borne rickettsial illness (CDC 2008), and prevention requires tick control. Humans and dogs can be affected, and clinical signs of infection include high fever, erythmic rash, anorexia, vomiting, diarrhea, injected haemorrhagic mucous membranes and lymph node tenderness (Biberstein and Zee 1990). This disease has not been reported in Australia.

Rickettsial diseases found in Australia include murine typhus (*Rickettsia typhi*), scrub typhus (*Orientia tsutsugamushi*), Queensland tick typhus (*Rickettsia australis*), and Flinders Island spotted fever (*Rickettsia honei* strain "marmionii") (Unsworth 2007). The latter two diseases are endemic to the Torres Strait Islands. All four diseases have similar clinical manifestations, which may include maculopapular rash, fever, headache, rigor, myalgia, and

arthralgia. Laboratory investigation is therefore required to identify the specific rickettsial etiologic agent (Lane *et al.* 2005)

Scrub typhus (*Tsutsugamushi* Fever) is typically acquired when infected chiggers, the larval stage of trombiculid mites (*Leptotrombidium deliense)* and others, bite whilst feeding to inoculate rickettsiae (Raoult 2005). The bacteria then multiply at the inoculation site with the formation of a papule that ulcerates and becomes necrotic. This lesion evolves into an eschar, with a regional lymphadenopathy, progressing to a generalized lymphadenopathy within a few days. Scrub typhus has also been diagnosed as the cause of death of a 38 year old park worker in Queensland (Pritchard 1988). Presenting signs included high fever, headache, myalgia and malaise. Following experimental infection scrub typhus has also been found to sub-clinically infect the short-nosed bandicoot, (*Isoodon macrourus)* (Heaslip 1941). Serum was surveyed from bandicoots in southern Queensland and agglutins found to *Rickettsia tsutsugamushi.* Further mouse inoculation, blood culture and agglutination tests confirmed infection. This led Heaslip (1941) to comment that the short-nosed bandicoot was a natural reservoir for scrub typhus.

North Queensland tick typhus, (*Rickettsia australis*), is found on the east coast of Australia and is spread by ticks belonging to the *Ixodes* genus and in particular *Ixodes holocyclus* (Lane *et al.* 2005). Humans are the primary host for this rickettsial species. However, the short-nosed bandicoot, brushtail possum *(Trichosurus vulpecula)* and rufous bettong (*Aepyprymnus rufescens)* have also been infected (Fenner 1946). *Ixodes holocyclus, Ixodes tasmani* and *Haemaphysasalis humerosa* were all found on affected animals. However isolation attempts were unsuccessful. *Ixodes tasmani* has been implicated in the transfer of North Queensland tick typhus to humans (Campbell and Domrow 1974). Yet owing to the behavioural nature and nesting activity of *Ixodes tasmani* the risk to humans of tick bite is low (Murdoch and Spratt 2005). Therefore its capacity as a vector to transmit zoonotic rickettsial disease is thought to be low.

Coxiella burnetii is the agent of Q fever, a zoonotic disease which occurs worldwide (Biberstein and Zee 1990). This rickettsial organism can be disseminated by the airborne route and principally affects domestic ruminants. Clinical signs of infection include placentitis and abortion. Humans are primarily exposed from a contaminated environment and clinical signs include influenza-like respiratory signs, although, mortality has occurred in cases of hepatic infection or endocarditis. Vaccination of female ruminants and humans at high risk of exposure is recommended. In marsupials, the short-nosed bandicoot, brushtail possum and rufous bettong have been experimentally infected with Q fever (Derrick *et al*. 1939) and natural infections were found to be common in wild bandicoots (Smith and Derrick 1940). Six strains of *Coxiella burnetti* were isolated from ticks *(Haemophysalis humerosa)* infesting short-nosed bandicoots on Moreton Island, Australia. Serum tested found agglutinins against *Coxiella burnetti*. Smith (1942) also found that all life stages of *Ixodes holocyclus* could be infected with *Coxiella burnetti* following feeding on infected laboratory animals and that the infection could subsequently be transmitted to bandicoots. *Ixodes holocyclus* also feeds on humans and so the potential for zoonotic infection is of concern. Derrick *et al.* (1939), Seddon (1951) and Derrick (1944) stated that bandicoots, especially the short-nosed bandicoot were an important reservoir of Q fever in Australia. The Gilbert's potoroo closely co-habits with the southern-brown bandicoot in Two Peoples Bay, and so spread via aerosolisation could certainly be a possibility.

4.1.2 Materials and methods

Captive individuals were trapped with the aid of a shade-cloth run, while wild potoroos were trapped in Sheffield cages. Initial health assessment was carried out via visual checking for faecal soiling, weight and body condition scoring. Optimal body condition was assigned to be between 2.5 and 3.5, with one being emaciated, and five obese (Chapter 2.1.3). If an individual was re-captured the animals's weight and body condition were compared to data from the prior trapping session. The Gilbert's potoroo was then anaesthetised and a

thorough physical exam was undertaken, in particular thoracic auscultation and ocular vitreal changes were closely monitored.

Blood was collected from the femoral vein and placed into an EDTA tube for a complete blood count (CBC) and also collected into a serum tube for biochemistry. The whole blood in the serum tube was centrifuged within four hours and a 0.5mL serum sample, as well as the EDTA sample were sent to Murdoch University via overnight courier on ice.

Each potoroo was thoroughly examined for ectoparasites and any associated pathology in the periocular regions, interscapula area, inguinal and axillary regions, surrounding the genitalia, in the pouch, around the base of the tail, and on the legs. This entailed careful brushing through the haircoat to allow a quantitative grading of overall parasite level to be assigned; encompassing mild (1+), moderate (2+) or heavy (3+) infestation. A representative selection of ectoparasites were removed with tweezers and stored in clear plastic vials for further identification to species level and life stage. Samples were batched and shipped to Murdoch University for parasite identification. Diagnosis was based on whole specimens examined under light microscopy using a Wild M3 stereomicroscope. Standard morphological criteria and dichotomous keys as described by Roberts (1970) were used for identification of genus and species.

In the November 2006 and March 2007 trapping sessions ticks were collected and forwarded to Mohammed Yazid Abdad, a PhD student at Murdoch University, for identification and PCR analysis for the rickettsial *glt*A gene. A positive result would indicate the presence of *Rickettsia* spp. bacteria in the tick. DNA extraction from arthropods and cell culture was performed using the QIAamp® DNA Mini Kit (Qiagen, Germany). Conventional PCR analysis was undertaken on all DNA extracts from materials suspected of harbouring rickettsial bodies including arthropods. Products were subsequently sequenced to

determine identity of the rickettsia detected in the samples. Gene targets used in the PCR assays were *gltA*, *ompA*, *ompB*, Gene D and *rss*.

Initial rickettsial isolation involved first identifying the arthropods. Standard morphological criteria and dichotomous keys as described by Roberts (1970) were used for identification of tick genus and species. Removal of potential contaminants was undertaken by immersing the arthropod in 95% sodium hypochlorite solution for approximately 10 minutes and then spraying with 70% ethanol and air drying in a level 2 biohazard hood. Arthropods were then cut into two with a sterile scalpel blade and placed into separate microcentrifuge tubes; one section to be used for PCR in 500µL of phosphate buffered saline (PBS) and the other for rickettsia isolation in 1mL of PBS. The section to be used for rickettsia isolation was crushed up with the blunt end of a sterile plastic inoculation loop (Sarstedt, Germany). Then 1mL of tick homogenate was filter sterilised with a 0.45µm filter (Millex, Ireland) attached to a sterile 3mL syringe (Turumo, The Philippines). The filter-sterilized homogenate was inoculated into 25cm^2 flasks containing monolayers of Vero, L929 and/or XTC-2 cells with 80-90% confluency. Flasks were then centrifuged at 600*g* for 20 minutes to induce entry of rickettsia into the cell monolayer. Cultures were incubated for up to two months at 35°C in 5% CO_2 (L929/Vero) or 28°C (XTC-2), with fortnightly media changes. Cultures were examined microscopically weekly and by IFA monthly. If deemed positive, cell cultures had their DNA extracted and the presence of rickettsia confirmed via PCR.

Each rickettsial PCR was performed using the same reaction protocols and cycling conditions with the exception of primer types, $MgCl_2$ concentrations and thermocycler annealing temperatures. These variables are summarized for each PCR performed (Table 4.1.2).

Table 4.2 Oligonucleotide primers $MgCl_2$ concentrations and thermocycler annealing temperatures used for rickettsial PCR amplification.

Primer	Nucleotide sequence	Gene	$MgCl_2$ concentration (mM)	Annealing temperature (°C)	Reference
RpCS877p	GGGGGCCTGCTCACGGCGG	*gltA*	3	51	Regnery *et al.*, 1991
RpCS1258n	ATTGCAAAAAGTACAGTGAACA	*gltA*	3	51	Regnery *et al.*, 1991
Rr190.70p	ATGGCGAATATTTCTCCAAAA	*ompA*	2	48	Regnery *et al.*, 1991
Rr190.602n	AGTGCAGCATTCGCTCCCCCT	*ompA*	2	48	Regnery *et al.*, 1991
120M59	CCGCAGGGTTGGTAACTGC	*ompB*	2	51	Roux *et al.*, 2000
120807	CCTTTTAGATTACCGCCTAA	*ompB*	2	51	Roux *et al.*, 2000
D1f	ATGAGTAAAGACGGTAACCT	*Sca4*	2	51	Sekeyova *et al.*, 2001
D928r	AAGCTATTGCGTCATCTCCG	*Sca4*	2	51	Sekeyova *et al.*, 2001

In addition to the primer types and $MgCl_2$ concentrations, each reaction contained 1μmol of each primer (Seraing, Belgium), 200μmol of each deoxyribonucleotide triphosphate (dNTP), 10× reaction buffer, 2U Taq polymerase (Invitrogen, Australia), 1.5μl of DNA extract and enough H_2O to make the final reaction volume 25μl. The amplification was performed in a Rotor-Gene 3000 thermocycler (Corbett Life-Sciences, Australia) with an initial denaturation of 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, 30 seconds of an appropriate annealing temperature and extension at 72°C for 1 minute. Following the cycling, a final extension at 72°C for 10 minutes was performed. PCR products were compared to known positive and negative controls by viewing under ultra-violet light on a 1% Tris acetate agarose gel (Sigma-Aldrich, USA) stained with ethidium bromide (Sigma-Aldrich, USA) electrophoresed for 30 minutes at 100V and visualized using an ultraviolet transilluminator.

PCR amplicons positive for rickettsial DNA were excised from their gels and purified using the QIAquick DNA clean up kit (Qiagen, Germany) and were sequenced at the State Agriculture and Biotechnology Centre (Perth, Western Australia). Sequencing reactions were performed using an ABI Prism Dye Terminator Cycle Sequencing Core Kit (Applied Biosystems, California) according to the manufacturer's instructions.

All sequenced products were then analysed using the computer program Chromas Lite (The University of Texas, USA) and were compared to sequence data available from GenBank, using the BLAST 2.2.14 program. Phylogenetic alignment of DNA sequences is still to be undertaken using the programme ClustalX. To calculate the genetic difference between the *Rickettsia* spp., phylogenetic analysis is to be conducted using Mega2 (using Tamura and Nei and neighbour- joining algorithms). This research is awaiting finance.

The statistics program SPSS® (Chicago, Illinois) was used to check for statistical associations between certain protozoal endoparasites and body condition. Odds ratios (OR) were used for the analysis of data when significant differences between groups were observed.

4.1.3 Results

No ectoparasites were found on captive potoroos. Fleas recorded in the wild population of Gilbert's potoroos included *Stephanurus dasyurii* and *Pygiopsylla* spp. Ticks recorded included adult and nymph stages of *Ixodes* spp. including *Ixodes australiensis, Ixodes fecialis* and *Ixodes myrmecobii*. In addition mites from the *Trombiculidae* family were identified.

Fleas were found throughout the haircoat of the potoroos in low numbers. Ticks were typically found in periocular regions, attached to the pinna and nasal planum, on the hocks and lower limbs, and around the cloaca. The trombiculid mites had a predilection for the sparsely haired regions including the inner thighs, and surrounding the scrotum. In severely affected individuals, severe inflammation, erythema and blood loss associated with the skin scrape was evident. The skin in these regions had diffuse raised orange encrusted mites disrupting the epithelium.

Only two potoroos were found to have severe inflammation associated with the presence of trombiculid mites. These were GP M55 and GP M116 in the November 2006 trapping session. Male GP 55 was trapped at Hakea trap one and GP M116 at North Firebreak trap number 16. These traps are approximately two km apart. Male 55 was an adult potoroo in sub-optimal body condition (condition score 2, weight 930g), he displayed bilateral hair thinning over the lateral flank regions with associated trombiculid mite infestation (Figure 4.3). This was an unusual presentation as the trombiculid mites tended to have a predilection for alopecic or minimally haired regions. (Figure 4.4).

Figure 4.3 Hair thinning over lateral flanks in male Gilbert's potoroo 55 (GP M55).

Sub-adult GP M116 was in sub-optimal condition for a wild individual (condition score 2, weight 950g) and was also severely infested with Trombiculids. No associated inflammatory response or peripheral eosinophilia was evident in either GP M55 or GP M116 as has been reported in severe parasitic infections in dogs. All other wild caught potoroos that had emerged from the pouch displayed a mild to moderate parasite burden with fleas, ticks, lice and mites. However, the proportion of potoroos displaying a moderate burden was highest in the March and November trapping sessions (Table 4.5).

Figure 4.4 Severe inflammation associated with Trombiculid infestation in GP M55.

Table 4.5 Seasonal ectoparasite burden in wild Gilbert's potoroos.

Month of year	March		June		November	
Year	2005	2007	2005	2006	2005	2006
Mild infestation	5/9 55.5%	10/17 58.8%	7/8 87.5%	6/7 85.7%	8/14 57.1%	2/9 22.2%
Moderate infestation	4/9 44.4%	7/17 41.2%	1/8 12.5%	1/7 14.2%	6/14 42.8%	5/9 55.5%
Severe infestation	0	0	0	0	0	2/9 22.2%
Total trapped	**9**	**17**	**8**	**7**	**14**	**9**

No potoroos were witnessed to be pruritic from the presence of ectoparasites. However, this should be interpreted carefully as wild potoroos were only briefly examined before being placed into the calico bag for anaesthesia and sample collection, and following the procedure when returned to the wild. Therefore pruritus, if present may not have been observed.

In total five *Ixodes australiensis* and two *Ixodes fecialis* were tested for *Rickettsia* spp. bacteria. *Rickettsia* spp. bacteria were found in one *Ixodes australiensis* collected. The speciation of this *Rickettsia* and serum testing for immunofluorescence is dependent on

acquisition of further funding. A novel *Ixodes* spp. tick was also discovered. This tick also requires further identification and speciation.

4.1.4 Discussion

The survival and abundance of ectoparasites is influenced by many factors, including the life stage of the parasite, environmental temperature and humidity, vegetation cover, and the presence of suitable hosts (Lorch, Fisher and Spratt 2007).

The proportion of potoroos displaying a moderate burden was highest in the March and November trapping sessions. This coincides with the more temperate weather patterns of autumn and spring as compared to the June trapping session which is winter and frequently cold, wet and windy. Ectoparasites are attracted by moist warm conditions. The paralysis tick thrives at an ambient temperature of 27 °C and high relative humidity (Clunies-Ross 1835). Very dry or very wet conditions are not ideal. The need for humid conditions largely determines the botanical niche of the tick. Low, leafy vegetation provides higher humidity levels by reducing the dessicating effects of wind and direct sunlight (Beveridge 1993).

Potoroos are ground dwellers, foraging for food and nesting in areas which ticks inhabit and therefore they would be constantly exposed to ticks in the warmer months of autumn and spring. This correlates with the increased prevalence of tick infestation in the March and November trapping sessions, and the lower prevalence found in the winter months (June trapping session). Other parasite hosts, such as reptiles, also hibernate during the colder, wetter winter months, limiting host numbers and subsequently minimizing the number of ectoparasites feeding on hosts to complete their reproductive life cycle.

This is the first report of *Ixodes australiensis, Ixodes fecialis* and *Ixodes myrmecobii* infecting Gilbert's potoroos. All three of these tick species have been reported previously in the south-west of Western Australia infecting various marsupial species. As such their

presence in this study is not surprising. It is however of interest that *Ixodes fecialis,* unlike the other two species, has not been previously reported as far south as the Albany district. This may be due to the fact that only limited sampling has been carried out this far south, or that the ticks are expanding their geographic range with the increasing interest in bushwalking and in particular the opening up of the Serpentine to Albany Bibbulmum track. Overabundance of feral animal populations, and dispersal of wildlife into new areas associated with land degradation and clearing, may also play a part in the observed expansion of this tick's geographic range.

This is the first report of the presence of rickettsiae in *Ixodes australiensis*. Serum testing for immunofluorescence will be undertaken to check the antigen titer of the infected individual once further funding becomes available. Positive results are typically reported for titres higher than and including 1:128. A cut off value of 1:128 is the recommended value given by the Australian Rickettsial Reference Laboratory (ARRL) to avoid misreads of cross-reacting antibodies. It is important to speciate the rickettsiae found as it represents the first rickettsia isolation in the Gilbert's potoroo. Furthermore, given that Gilbert's potoroos have been found to carry *Ixodes* spp. and *Trombiculids,* there may be the potential of potoroos being infected with the rickettsial agents that cause scrub typhus or Queensland tick typhus in humans. This is not only important with regards to developing further understanding about the biology and health of potoroos, but is also very significant from a human health perspective. *Rickettsiae* has yet to be isolated this far south in Western Australia and due to the number of bushwalkers and DEC staff that come into contact with ticks, further protective measures may need to be implemented.

4.1.5 Conclusion

Fleas were found throughout the haircoat of the potoroos in low numbers. Ticks were typically found in periocular regions, attached to the pinna and nasal planum, on the hocks and lower limbs, and around the cloaca. Trombiculid mites seemed to have a predilection

for the sparsely haired regions including the inner thighs, and surrounding the scrotum. In severely affected individuals severe inflammation, erythema and blood loss on superficial skin scrape was evident.

The proportion of potoroos displaying a moderate ectoparasite burden was highest in the March and November trapping sessions, coinciding with the more temperate weather patterns of autumn and spring compared to the winter June trapping session which frequently cold, wet and windy. Other reasons for mild winter infestations may include the lack of suitable parasite hosts, and hibernation of ectoparasites at this time.

This is the first report of *Ixodes australiensis, Ixodes fecialis* and *Ixodes myrmecobii* in Gilbert's potoroos. Additionally, a novel *Ixodes* spp. was identified and a novel *Rickettsia* spp. was isolated from an *Ixodes australiensis* tick.

GASTRO-INTESTINAL PARASITES

4.1.5 Introduction

Like the ectoparasites, current knowledge about gastro-intestinal parasites and associated host responses in Australian mammals is continually evolving. The majority of literature has relied on opportunistic sampling such as the collection of road kill or the collection of specimens via professional shooters (Oakwood and Spratt 2000). Systematic surveys are rare and most report on marsupials from the eastern states (Adams 2003). Life-cycles are inferred from related parasites of domestic animals and then further interpreted in relation to the relevant digestive anatomy and physiology of the species (Dunsmore 1976).

4.2.1.1 Helminth infection

A dominance of helminth flora has been reported in Australian marsupials (Beveridge and Spratt 1996, Spratt 1997). Of the helminths the trematoda are represented by nine families, the cestoda by five families, the Acanthocephala by two genera and the nematodes with up to 20 families (Beveridge and Spratt 1996).

In macropods, the majority of surveys conducted have reflected a dominance of strongyloid nematodes (Beveridge and Arundel 1979). In particular, kangaroos have a marked diversity of nematodes. A total of more than 30 000 species of nematodes have been identified (Beveridge and Arundel 1979). Dasyurids and potoroids also show a dominant helminth burden (Spratt 1987). However, researchers have only reported a sparse population of strongyloid nematodes in potoroids (Beveridge 1996) which has been related to their functional and digestive anatomy. The Gilbert's potoroo as a member of the *Potoroidae* family has a gastrointestinal tract consisting of a large sacculated forestomach, with minimal development of the tubiform forestomach and a small hindstomach (Beveridge 1996). The large sacciform forestomach is thought to have evolved as a storage organ rather than a region of fermentative digestion (Frappel and Rose 1986). The caecum and colon are the sites for the majority of fermentative digestion (Hume 1982) although Carr (1970) demonstrated fermentation also occurred in the forestomach. This contrasts to macropods where fermentative digestion occurs primarily in the stomach. This may account for the lack of strongyloids in the stomachs of potoroos compared to macropods. The strongyloidea typically reside burrowed in the tissue of the forestomach prior to dissemination to the large bowel. It has been proposed that the limited formation of volatile fatty acids in the stomach of potoroos due to fermentative digestion primarily occurring in the caecum has resulted in a reduced fauna of strongyloidea (Adams 2003). The lack of strongyloids in the stomach reducing the diversity of helminth species recorded in potoroid species (Beveridge *et al.* 1992).

4.2.1.2 Pathogenic effects, diagnosis and treatment of helminths

Hypodontus macropi has caused death in captive red kangaroos through localised haemorrhage and enteritis from parasite attachment (Arundel 1977). *Globocephaloides* spp. has caused death in wild eastern-grey kangaroos associated with a severe anaemia and hypoproteinemia (Arundel *et al.* 1977). This parasite is thought to have a hookworm-like pathogenicity (Beveridge *et al.* 1994). Strongyloidiasis has been implicated in deaths of captive macropods presenting with weight loss, progressive anorexia and death (Blyde 1999). Diagnosis of strongyloidiasis is conducted via faecal flotation using the Baermann technique to allow separation of the active larvae from the faecal mass given the lack of eggs passed in the faeces (Blyde 1999). Treatment of helminth infections involves anthelminthics, such as Ivomec (Ivermectin, Merial Australia) at a dose rate 200µg/kg and Cydectin® (Moxidectin) 200-500µg/kg orally, topical or parenterally, or benzimidazoles. Reported toxicity has occurred with the use of benzimidazoles in some species (Blyde 1994). Prevention centres on hygiene, limiting access to faecal matter, and quarantine treatments.

4.2.1.3 Protozoal infections

Protozoal infections in Australian marsupials have been sparsely recorded. This may be because growth characteristics, serology and host range are required for identification and speciation (Monis 1999). Coccidiosis caused by *Eimeria* spp. is the most commonly described protozoal disease in marsupials. Some macropods including the eastern-grey and western-grey kangaroos seem highly susceptible, especially in the event of stress, such as hand rearing, pouch emergence, or recent introduction into a new group of animals in captivity (Blyde 1999). Black striped and whiptail wallabies also seem susceptible (Speare *et al.* 1982).

4.2.1.4 Pathogenic effects, diagnosis and treatment of protozoans

Intestinal coccidia in macropods predominantly infect the cells of the mucosa of the small intestine (Speare *et al.* 1989). Coccidian oocysts are commonly found in the faeces of

captive macropods, however the associated disease state of coccidiosis is uncommon, occurring predominantly in juveniles (Speare 1988).

The protozoal disease, coccidiosis, may present with depression, lethargy, dysentery, anorexia, sudden weight loss from dehydration, profuse black malodorous diarrhoea or occasionally sudden death (Speare *et al.* 1992). Two forms exist; in the acute form death ensues within 24 hours with scant, black haemorrhagic diarrhoea being voided whereas in the sub-acute form death occurs within 48-72 hours (Blyde 1999). Diagnosis relies upon correlating clinical signs with the presence of oocysts in the faeces observed through faecal flotation. Treatment involves use of anti-protozoals, including sulphonamides such as amprolium and toltrazuril (Baycox® at 25mg/kg PO s.i.d for three days), and trimethoprim and sulfonamide combinations (Tribrissen® at 40mg/kg) in conjunction with fluid therapy and anti-diarrhoeals. Booth (1999) recommends the consideration of euthanasia if signs are severe. Prevention centers on hygiene, the maintenance of clean yards, placing feed in racks raised above the ground, and not overcrowding to minimise stress (Christian 1988).

4.2.1.5 Gastro-intestinal parasites of Western Australian marsupials

Adams (2003) reported on gastro-intestinal parasite populations of the following native West Australian marsupials, the brush-tailed bettong, brushtail possum, western-grey kangaroo, tammar wallaby (*Macropus eugenii)*, western quoll (*Dasyurus geoffroii)*, southern-brown bandicoot and the western-barred bandicoot (*Perameles bougainville)*. This study found that (59.1%) of native mammals sampled (n=808) were predominantly infected with unidentified nematodes of the order Strongylida. Members of the orders Rhabditida, Spirurida and Oxyurida were also common. Oxyurid nematodes were identified in 27.8% of western-grey kangaroos (n=35). Several species of *Eimeria* were detected in the marsupials. In the southern-brown bandicoot, a resident of Two Peoples Bay, *Eimeria potoroi, Eimeria gaimardi,* and *Eimeria aepyrmn*i were found. Unidentified species of Entamoeba and coccidia were also commonly reported in the marsupials studied.

4.2.1.6 Gastro-intestinal endoparasites of the long-nosed potoroo

The helminth flora of the analogous long-nosed potoroo was studied by Beveridge (1996). Small numbers of trichostrongyloid, strongyloid and oxyurid nematodes, with a few cyclophyllidean cestodes were reported. These consisted of two cestode species *Calostaurus mundayi* and *Potorolepsis potoroi,* the nematode *Potostrongylus finlaysoni* and the oxyurid *Potoxyuris potoroo.* The two cestode species utilise arthropods as an intermediate host, and it has been suggested that the ingestion of arthropods via foraging plays an important role in their infection with this parasite (Beveridge 1996). *Gongylonema* spp. have also been isolated from the long-nosed potoroo, agile wallaby, and brush-tailed possum (Beveridge 1978).

4.2.1.7 Specific gastro-intestinal parasites in Gilbert's potoroo

Prior to this study, limited information existed with regards to gastro-intestinal parasites of Gilbert's potoroos. An aged female wild potoroo in poor body condition was found dead, assumed to have been regurgitated by a carpet python in June 2004. In the thorax, multiple roundworms up to 10cm in length were found. One had penetrated the diaphragm with half its body in the thoracic cavity, and the other half in the peritoneal cavity. In the liver, multiple white nodules up to 0.5cm were present within which were coiled roundworms. In the peritoneal cavity, multiple roundworms, some in a fine fibrin mesh over a segment of haemorrhagic small intestine were also found. The roundworm was identified to be *Ophidascaris robertsi.*

The larval stages of *Ophidascaris robertsi* were also found on post-mortem examination in cysts in the liver of captive GP M10 (Horwitz and Forshaw 2001, unpublished data). The method of infection was assumed to be via the ingestion of python faeces (Horwitz and Forshaw, 2001, unpublished data).

The Australian carpet python, is the definitive host of the nematode *Ophidascaris robertsi* however a broad spectrum of rodents, reptiles and marsupials including the brushtail

possum, mountain brushtail possum *(Trichosurus caninus)*, sugar glider (*Petaurus breviceps)* and the marsupial glider (*Schoinobates volans)* (Booth 1999) may serve as intermediate hosts of this roundworm (Sprent 1963a, 1963b) This parasite has also been found to cause sub-clinical infections in echidnas, which are thought to be a dead-end host (Booth 1999). In the intermediate host, *Ophidascaris robertsi* eggs can take four weeks to develop to third-stage larvae, which are infective for the carpet python (Sprent 1963b). If the intermediate host has been infected for four or more weeks and is then eaten by a carpet python, ingested third-stage larvae will moult and complete their development to adults in the wall of the stomach and oesophagus of the definitive host (Sprent 1963b).

A total of 54% of mountain brushtail possums examined on post-mortem at Clouds Creek in north-eastern New South Wales had lesions associated with infection by *Ophidascaris robertsi* (Presidente *et al.* 1982). The larvae were weighed and found to comprise up to 30-40% of the total weight of the liver and caused extensive damage to the hepatic parenchyma, including focal cholangitis and dilatation and fibrosis of bile ducts that contained third-stage larvae (Sprent 1963b; Presidente *et al*. 1982). Interestingly however, a female with 216 *0phidascaris robertsi* larvae remained in good body condition (assessed from internal fat deposits) and was reproductively active despite extensive liver damage (Presidente *et al.* 1982).

Although the primary pathogenicity of *Ophidascaris* remains questionable, current husbandry practices have been developed to attempt to exclude all python faeces from the captive colony enclosures (Horwitz and Forshaw, 2001, unpublished data). The female described above also had a 15cm segment of terminal small intestine that appeared grossly haemorrhagic with blood-tinged, pasty contents within which numerous thin roundworms (approximately 1cm in length) were found. These were identified to be *Potorostrongylus temperatus.* The associated inflammatory reaction was indicative of the pathogenicity of this species. *Potostrongylus temperatus* has only been recorded previously in the eastern

bettong (*Bettongia gaimardi)* in Tasmania (Smales 1997). This is interesting as Smales (2005) postulated that each potoroid host species seems to be associated with a particular species of potorostrongylus, suggesting co-evolution of host and helminth, and additionally that potoroid-potorostronglyid associations appear to be incidental infections resulting from the genetic divergence of multiple populations (from a single parent species) inhabiting the same geographic region (sympatric speciation). However, this does not seem to be the case as an associated inflammatory response was found in the Gilbert's potoroo infected with *P. temperatus* and eastern bettongs, which exist in a geographically distinct area are also reported hosts of this parasite.

4.2.2 Materials and methods

Trapping, initial health assessment, anaesthesia, physical exam and blood collection of the wild and captive population was described in 4.1.2. Faecal samples were collected from the floor of the captive potoroos enclosure or the Sheffield cage using surgical forceps and placed into a labeled zip lock bag on ice. Samples were then couriered to Murdoch University overnight on ice for faecal concentration and parasite identification. Helminth and protozoan parasites were detected using zinc sulphate flotation methods to concentrate eggs and cysts and viewed via light microscopy at 10x and 40x magnification. Identification of parasite species was based on egg and cyst morphology. The majority of eggs could only be identified to a family or order level.

4.2.3 Results

Instead of analysing each trapping session separately it was decided to combine all data to indicate the prevalence of certain gastrointestinal parasites over the entire study period from March 2005 to March 2007. This meant that the data analysed included total number of faecal samples collected, including re-sampling of both captive and wild individuals. However, only one faecal sample from trapped potoroos was collected for screening during

each trapping session. This technique was chosen given the small population size of the Gilbert's potoroo and to ensure that many of the endoparasites present in only low numbers were represented in the population data. It was also thought appropriate as only one individual was thought to have gastro-intestinal signs associated with the parasitism. In total 71 faecal samples were analysed from 37 individuals, comprising 30 wild and seven captive individuals (Appendix 3). No parasitic organisms were detected in eight faecal samples, from the captive population.

A predominance of strongyle eggs was found, 52.1% of the population had strongyle eggs in their faeces at one point during the course of the study. Unidentified nematode larvae were found in 30.9% of the population, 2.8% of the population showed infection with *Trichuris* sp. and 1.4% had unidentified nematode eggs.

An entire potostrongylus worm was found on faecal examination of wild GP M83 in June 2006. The faeces were loose and pungent smelling and faecal soiling was evident. Body condition was 2.5 (optimal), blood parameters were unremarkable and a cloacal swab revealed a moderate mixed growth of coagulase-positive *Staphylococcus* spp. and alpha-haemolytic *Streptococcus*, both commonly isolated microbes. On faecal examination, during the subsequent trapping period six months later no other potostrongylus worms were detected. Strongyle eggs and nematode larvae which had been present throughout all prior trapping sessions without clinical effect were still evident.

There was a higher or equal prevalence of mild endoparasitic infection (1+) with nematodes in male Gilbert's potoroos compared to females (Table 4.6). At moderate levels of infection (2+) females had a higher prevalence of strongyle eggs and nematode larvae compared to males. There was only one male and one female found to have a severe infection (3+) with strongyle eggs.

Table 4.6 Nematode prevalence in male compared to female Gilbert's potoroo.

	Nematodes									
Parasitic burden in Gilbert's potoroos (n=37)	***Trichuris* sp.**		**Strongyle eggs**		**Strongyle Larvae**		**Unidentified nematode eggs**		**Unidentified nematode larvae**	
Sex affected	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
1+	1/71 1.4%	1/71 1.4%	19/71 26.7%	14/71 19.7%	3/71 4.2%	2/71 2.8%	1/71 1.4%	0	13/71 18.3%	8/71 11.3%
1 + (total)	2/71 2.8%		33/71 46.4%		5/71 7%		1/71 1.4%		21/71 29.5%	
2+	0	0	0	2/71 2.8%	1/71 1.4%	0	0	0	0	1/71 1.4%
2 + (total)	0		2/71 2.8%		1/71 1.4%		0		1/71 1.4%	
3+	0	0	1/71 1.4%	1/71 1.4%	0	0	0	0	0	0
3 + (total)	0		2/71 2.8%		0		0		0	
Total prevalence	2/71 2.8%		37/71 52.1%		6/71 8.4%		1/71 1.4%		22/71 30.9%	

When assessing nematode prevalence in the captive compared to the wild population (Table 4.7) strongyle eggs, strongyle larvae and unidentified nematode larvae were found in both populations. *Trichuris* sp. was only found in the captive population. Unidentified nematode eggs, were only found in one wild individual.

Strongyle eggs were the most prevalent nematode found in both the captive and wild population, with 54.9% (28/51) of wild individuals having a mild parasitic burden (1+). Correspondingly 20% (4/20) of captive individuals had a mild parasitic burden. Unidentified nematode larvae followed proportionally with 15% (3/20) of captive individuals and 37.2% (19/51) of wild individuals having a mild parasitic burden. Strongyle larvae then followed with 5% (1/20) of captive individuals and 7.8% (4/51) of wild individuals showing a mild parasitic burden.

Table 4.7 Nematode prevalence in the captive compared to the wild population.

	Nematodes				
Parasitic burden in Gilbert's potoroos (n=37)	***Trichuris* sp.**	**Strongyle eggs**	**Strongyle Larvae**	**Unidentified nematode eggs**	**Unidentified nematode larvae**
Captive Population (n=7) 20 samples					
1+	2/20 10%	4/20 20%	1/20 5%	0	3/20 15%
2+	0	1/20 5%	0	0	0
3+	0	0	0	0	0
Wild Population (n=30) 51 samples					
1+	0	28/51 54.9%	4/51 7.8%	1/51 1.9%	19/51 37.2%
2+	0	1/51 2%	1/51 2%	0	1/51 2%
3+	0	2/51 3.9%	0	0	0

Strongyle eggs were present in moderate (2+) to large (3+) numbers. The wild population had the highest prevalence of heavy strongyle egg infection, despite being found in only 3.9% of the population. Strongyle eggs, larvae and unidentified nematode larvae were all present in moderate numbers in the wild population 2% (1/51). No oxyurids were detected.

Body condition and associated parasite burden was examined. (Table 4.8). Of the 37 individuals which had faecal samples examined for gastro-intestinal parasites, 19 were in sub-optimal body condition (less than or equal to body condition score 2). These were all wild individuals which did not alter their body condition score over the testing period. Captive individual body condition scores ranged from 3.5 to 5, and fluctuated over the testing period.

Two potoroos had sub-optimal body condition scores of 1.5 and one potoroo was classified as emaciated with a body condition score of 1. In general, there was a low prevalence of moderate (2+) to heavy (3+) infection. In the total population helminth parameters, a predominance of strongyle eggs was found, with all potoroos in sub-optimal body condition having a mild burden (1+) of strongyle eggs (100%). Eleven (68.7%) of individuals with a

body condition score of 2 had unidentified nematode larvae present, and only one individual with unidentified nematode larvae was in a body condition score of 1.5.

Table 4.8 Nematode infection in relation to sub-optimal body condition in captive and wild Gilbert's potoroo.

	Nematodes				
Parasitic burden in Gilbert's potoroos	***Trichuris* sp.**	**Strongyle eggs**	**Strongyle Larvae**	**Unidentified nematode eggs**	**Unidentified nematode larvae**
Body condition score 2 (n=16)	0	16/16 100%	0	0	11/16 68.7%
Body condition score 1.5 (n=2)	0	2/2 100%	0	0	1/2 50%
Body condition score 1 (n=1)	0	1/1 100%	0	0	0

Overall, the highest prevalence of mild nematode endoparasitic infection (1+) was seen over the June-July trapping session where 44.5% of isolates were nematode parasites (37/83). In the November-December trapping session 34.9% of the population had a mild nematode burden and this declined further to 20.4% in the March-April trapping session. The moderate and severe nematode infections were not compared due to their small sample sizes and the corresponding lack of statistically significant data which would be obtained.

The highest prevalence of mild strongyle egg infection was seen over the November-December trapping session (72.4%), whereas the lowest prevalence of infection was seen in the March-April trapping session (41.2%) (Table 4.9). Strongyle larvae prevalence was highest (23.5%) over the March-April trapping session. Unidentified nematode eggs were only found in the March-April trapping session and were in low prevalence (5.8%). Unidentified nematode larvae had the highest prevalence of mild infection during the June-July trapping session (40.5%).

Table 4.9 Seasonal spread of nematode infection in captive and wild Gilbert's potoroo.

Parasitic burden in Gilbert's potoroos (n=37)	*Trichuris* sp.	Strongyle eggs	Strongyle Larvae	Unidentified nematode eggs	Unidentified nematode larvae
1 + infection 83 nematode isolates	2/83 2.4%	49/83 59.1%	5/83 6%	1/83 1.2%	26/83 31.3%
March-April (n=17)	0	7/17 41.2%	4/17 23.5%	1/17 5.8%	5/17 29.4%
June-July (n=37)	1/37 2.7%	21/37 56.7%	0	0	15/37 40.5%
Nov-Dec (n=29)	1/29 3.4%	21/29 72.4%	1/29 3.4%	0	6/29 20.7%

4.2.2.1 Protozoans

The highest prevalence of infection with protozoans was of unidentified coccidian oocysts, 11.3% had mild infections (1+) of unidentified oocysts at one point during the course of the study. (Table 4.10). This was followed by *Eimeria* infection 5.6% of the population had mild numbers (1+) of this organism at one point during the course of the study. Interestingly, the wild population seemed to have a higher prevalence of infection with unidentified coccidian oocysts 13.7% (7/51) compared to the captive population 5% (1/20). *Eimeria* had a 10% 2/20 prevalence in the captive population and 3.9% (2/51) prevalence in the wild population. Infection was always mild, no moderate or heavy protozoal burden was found.

Males had a higher prevalence of mild endoparasitic burden (1+) with protozoans compared to females.

Table 4.10 Protozoan infection in males compared to females, and the captive compared to the wild population of Gilbert's potoroo.

Protozoa				
Parasitic burden in Gilbert's potoroos (n=37) 71 faecal samples	***Eimeria***		**Unidentified coccidian oocysts**	
Sex affected	**M**	**F**	**M**	**F**
	3/71 4.2%	1/71 1.4%	5/71 7.1%	3/71 4.2%
1 +	4/71 5.6%		8/71 11.3%	
2+	0		0	
3 +	0		0	
Captive Population (n=7) 20 samples				
1+	2/20 10%		1/20 5%	
2+	0		0	
3+	0		0	
Wild Population (n=30) 51 samples				
1+	2/51 3.9%		7/51 13.7%	
2+	0		0	
3+	0		0	

Weight, body condition and associated parasite burden was also examined in relation to protozoal burden (Table 4.11). No faecal protozoal infection was recorded in individuals of body condition score 1.5 or 1. Only 6.2% (1/16) of individuals with a sub-optimal body condition of 2 had an *Eimeria* burden. While 18.7% (3/16) of individuals with a sub-optimal body condition of 2 had a mild burden of unidentified coccidian oocysts. SPSS descriptive statistic functions were used to analyse odds ratios for *Eimeria* and unidentified coccidian oocyst infection in relation to sub-optimal body condition in those individuals infected with protozoans. Sub-optimal body condition was assigned to be (0) and optimal body condition (1). A statistically significant relationship was not found between individuals with *Eimeria* infection that were in sub-optimal body condition ($p=0.424$). Compared to those in optimal body condition (condition score 2.5 or above). Likewise, a statistically significant relationship was not found between individuals with coccidian infection that were in an sub-optimal body condition, compared to those in optimal condition ($p=0.424$). This suggests that *Eimeria* and unidentified coccidian oocyst infections are not linked to sub-optimal body condition.

Table 4.11 Protozoal infection in relation to sub-optimal body condition in captive and wild Gilbert's potoroo.

	Protozoa	
Parasitic burden in Gilbert's potoroos	***Eimeria***	**Unidentified coccidian oocysts**
Body condition of Gilbert's potoroos	4/37 10.8%	8/37 21.6%
Body condition score 2 (n=16)	1/16 6.2%	3/16 18.7%
Body condition score 1.5 (n=2)	0	0
Body condition score 1 (n=1)	0	0

Overall the highest prevalence of mild protozoal infection was seen over the June-July trapping session (Table 4.12). Unidentified coccidian oocysts were most prevalent in the June-July (18.9%) trapping session, whereas *Eimeria* had a 11.7% prevalence in both the March-April and June-July trapping sessions.

Table 4.12 Protozoal infection in captive and wild Gilbert's potoroo in relation to season.

	Protozoa	
Parasitic burden in Gilbert's potoroos (n=37)	***Eimeria***	**Unidentified coccidian oocysts**
Season	4/37 10.8%	8/37 21.6%
March-April (n=17)	2/17 11.7%	0
June-July (n=37)	2/37 5.4%	7/37 18.9%
Nov-Dec (n=29)	0	1/29 3.4%

4.2.3 Discussion

Parasites which are reportedly non-pathogenic can become pathogenic if stress occurs in the host due to factors such as overcrowding, malnutrition, age, increased density of infective stages, and environmental change (Scott 1988). Increased density leading to increased transmission rates of density dependent parasites may also occur.

This study has investigated the gastro-intestinal parasites of the Gilbert's potoroo and identified parasitic species commonly isolated in an attempt to define a 'normal' parasite burden, and to compare captive to wild potoroos' parasitic burdens. In the captive colony, potoroos are treated every three months with pyrethrin and ivermectin to eradicate parasites. This is based on the premise that increased densities of infective stages in captivity may weaken the body's normal immune response and that wild individuals coming into captivity may transmit parasites to the captive population. The captive potoroos are kept in an environment which is relatively free of parasites in an attempt to provide an optimum environment for breeding and survival. In the short term, if breeding is successful, this is advantageous for the survival of the species. However long term, these captive-bred individuals if ever released, may be highly susceptible to parasitic infection. Infectious disease is a normal feature of the life of most organisms. Parasite elimination can weaken a population, however control and management can enable a population to function optimally. This is attained in the captive population by controlling vectors of endoparasites, soil changes to minimize the build up of infective stages and prophylactic treatment. This ensures gastro-intestinal parasites have minimal impact on the health of captive animals.

Overall, a dominance of strongyloid nematodes was seen with 60.5% of the total population having a mild infestation (1+) over the course of the study. This result was unsurprising given the two common marsupial species found in the Two Peoples Bay Nature reserve, the western-grey kangaroo, and the southern-brown bandicoot also reportedly had prevalences of strongyloid nematode infections of 88.9% and 68.8% respectively (Adams 2003). Unidentified nematode eggs, were only found in one wild individual and *Trichuris* sp. was only found in the captive colony. This may relate to the direct life-cycle of *Trichuris* sp. with re-infection more likely given constant habitation and only intermittent substrate changes and anthelmintic administration in the captive colony.

Adams (2003) reported a high prevalence of *Trichuris* sp. infection in captive bandicoots given routine anthelmintic treatment with 200μg/kg of ivermectin. It was proposed that such treatments did little to eliminate the infective stages and so re-infection occurred exacerbated by the infrequent changing of the soil and substrate. The only species known to infect marsupials, *Trichuris peremalis,* has been reported in several species of bandicoot genera (Beveridge and Spratt 1996). Southern-brown bandicoots are found in close proximity to potoroos on Mount Gardner and share a similar habitat. There is a possibility of transfer occurring when wild potoroos forage through detritus for food and come into contact with contaminated bandicoot faeces, however, faecal flotation examination found no evidence of infection in wild potoroos. In captivity, it would be assumed that the potoroos that remained infected despite anthelmintic treatment were in enclosures where inadequate elimination of environmental infective stages, or inadequate administration of anthelmintic had occurred.

Strongyle eggs were the most prevalent nematode found in both the captive and wild population, with 54.9% (28/51) of wild individuals having a mild parasitic burden (1+). Correspondingly, 20% (4/20) of captive individuals had a mild parasitic burden. The wild population also had the highest prevalence of heavy strongyle egg infection (3+). This was the only heavy infestation found in both the wild and captive population. Strongyle eggs, larvae and unidentified nematode larvae were all present in moderate numbers in the wild population 2% (1/51). The higher levels of infection present in the wild compared to the captive population can probably be explained by the routine anthelminthic treatment applied to the captive population, however the presence of low grade infestation with strongyle eggs, strongyle larvae and unidentified nematode larvae in the captive population would suggest that current anthelmintic treatment is ineffective. This may be due to the ineffective administration of required dose, infrequent application, resistance to the drug, or increased densities of infective stages (Scott 1988). Reinfection owing to infrequent removal of substrate would be another possibility.

Overall the highest prevalence of mild nematode and protozoal endoparasitic infection was seen over the June-July trapping session. Life cycle and conditions favouring dispersal have been inferred from domestic species models in this chapter due to the lack of other suitable data. Climatically, June-July is the wettest time of the year in Two Peoples Bay and the higher prevalence of infection may reflect increased dispersal of infective larval stages with moist conditions. This phenomenon is frequently seen in the sheep and cattle industry in southern Australia where the number of nematode larvae on pasture increases with the onset of rain (June), peaks in August/September and decreases to undetectable levels in the dry season (Arundel 1989). Differences in grazing and vegetation type exist when comparing domestic ruminants to wild Gilbert's potoroos, however, given the highest number of nematodes 44.5% (37/83) were isolated in the wet winter season, a similar pattern of infection appears present. Likewise, Arundel *et al.* 1990 reported increased numbers of *Strongyloides* sp. in marsupials in wet seasons, raising the possibility of nematode disease in particularly wet years. The possibility of other hosts maintaining these parasites should also be considered. Adams (2003) reported an 88.9% prevalence of nematodes in western-grey kangaroos and a 60% prevalence in southern-brown bandicoots in Western Australia. Both of these species are present within the Two Peoples Bay Nature Reserve. All of these hosts could theoretically shed infective stages, contaminate the detritus and heath where the potoroos forage and dig, establishing an ongoing cycle of infection and contributing to endemic low levels of nematode infection.

Only wild individuals were in sub-optimal body condition. All wild potoroos in sub-optimal body condition had a mild burden (1+) of strongyle eggs (100%). While there are numerous factors which may account for the sub-optimal body condition observed, the presence of strongyle eggs in all members of the wild population, and an associated lack of evidence of faecal soiling or visual evidence of endoparasitic disease may suggest that the wild population of Gilbert's potoroo has adapted to a low level of infestation with strongyles. In small populations susceptibility to pathogens is increased when the population size falls

below a critical level required to maintain disease (Cunningham 1996). These populations risk becoming immunologically naïve as many individuals will not be exposed, leading to low levels of acquired immunity (Viggers *et al.* 1993). This does not seem to be the case with regards to gastrointestinal parasite infection in the wild population. However, this could be a consideration prior to translocation. If translocated captive potoroos are immunologically naïve, or have received long term treatment for parasitism, they are at increased risk of being adversely affected by epidemics of previously endemic diseases (McCallum and Dobson 1995).

The one potentially pathogenic strongyle identified was the *Potostrongylus* worm found on faecal examination of wild GP M83 in June 2006. Clinically this individual displayed loose and pungent smelling faeces, and faecal soiling around the cloaca. Blood parameters were unremarkable and a cloacal swab revealed a moderate mixed growth of coagulase positive *Staphylococcus* spp. and alpha-haemolytic *Streptococcus*, both commonly isolated bacteria thought to be part of normal intestinal microflora. On subsequent faecal examination four months later, no other *Potostrongylus* worms were detected, faecal consistency had returned to normal and there was no evidence of faecal soiling. Strongyle eggs and nematode larvae which were present throughout prior trapping sessions without obvious clinical effects were also seen subsequently. This finding, in addition to the post mortem findings of the aged female regurgitated by the carpet python, suggest that *Potostrongylus* is pathogenic in Gilbert's potoroos.

The predominant coccidian found in two Western Australian marsupials endemic to the Two Peoples Bay Nature Reserve, namely the western-grey kangaroo and the southern-brown bandicoot, belong to the genus *Eimeria* (Adams 2003). The Gilbert's potoroo population had a 5.6% prevalence with 1+ (mild *Eimeria* infection) while 11.3% of the population were infected with unidentified coccidian oocysts at any one point during the course of the study. Adams (2003) reported that unidentified coccidians were detected in 16.7% of western-grey

kangaroos, and the majority of these were thought to be *Eimeria* species even though they remained unidentified. The lack of suitable molecular diagnostic tools was thought to hamper identification of the protozoans such that identification by traditional methods including host range, growth characteristics or serology was required, often leading to incomplete identification and classification (Monis 1999). Therefore it is possible that the prevalence of *Eimeria* in Gilbert's potoroos is in fact higher than reported.

The captive population seemed to have a higher prevalence of infection with *Eimeria* than the wild population. However, overall the prevalence of *Eimeria* was low, with a 10% (2/20) prevalence in the captive population and 3.9% (2/51) prevalence in the wild population. This is interesting given that *Eimeria* is a coccidian, with a direct life cycle which becomes particularly prevalent during times of immunosuppression. Only low levels of infection were found (1+) and no overt signs of clinical disease were seen, suggesting that the potoroo has adapted to low levels of infection.

However given the low prevalence of *Eimeria* infection in potoroos in the wild compared to the captivity, further consideration should be given to potential pathogenicity of this infection when wild individuals are brought into the captive colony. This inevitably will be a stressful period for the potoroo and it may be prudent to start coccidiostats, in conjunction with routine anthelmintic treatment, to guard against the oocysts shed from current captive occupants infecting a new member of the captive colony causing clinical disease. The recently invoked 14 day quarantine period for a new member of the captive colony may also help to minimise the potential for coccidial disease transfer.

No protozoal infection was recorded in individuals of sub-optimal body condition score 1.5 or 1. Only 25% (1/4) individuals with *Eimeria* had a sub-optimal body condition, likewise only 37.5% (3/8) of individuals with a mild burden of unidentified coccidian oocysts were in sub-optimal body condition. When the population infected with protozoal infection was analysed

in SPSS no statistical differences between sub-optimal or optimal body condition was found. This suggests that *Eimeria* (p=0.424) and unidentified coccidian oocyst infections (p=0.424) are not linked to sub-optimal body condition.

Given that coccidian oocysts are not fragile and can survive for weeks in a desiccated condition, daily cleaning of enclosures is undertaken in the captive colony to remove all faeces and faecal contaminated food and water. Insects found in the enclosure are also removed on a daily basis as they are a source of ants and act as another source of contamination (e.g. crickets and beetles may eat the oocysts while gathering fluid from the faeces).

The finding of unidentified coccidian oocysts in 11.3% of the population is higher than the 5.6% found in western-grey kangaroos and 4.3% found in southern-brown bandicoots in a Western Australian survey (Adams 2003). This is interesting given the direct life cycle of coccidians and the shared habitat the above species have with potoroos on Mount Gardner. A potential transmission route could involve the ingestion of contaminated soil when foraging for food and digging through detritus for truffles. Small and fragmented populations have also been reported to enhance transmission of direct life cycle parasites (Scott 1993, Viggers *et al*. 1993). As such, the higher prevalence of protozoans in the Gilbert's potoroo compared to other con-specific marsupials on Mount Gardner may reflect the fragmented and localized distribution of the Gilbert's potoroo.

Overall, it seems that current anthelmintic practices in the captive colony are not effectively ridding the population of nematodes. However, there are no clinical signs of disease and all captive individuals are well conditioned (body condition score 3.5-5). In fact their low nematode burdens may in fact be advantageous if these individuals are to be eventually released back into the wild. However, the lack of efficacy of treatment should be further investigated to ensure this low level of infestation is not linked to drug resistance otherwise

there is the potential to introduce a drug-resistant parasite which could cause epidemic disease in a wild population, immunologically naïve to this parasite. For this reason it is recommended that captive individuals are not released back into the wild population after undergoing an anthelmintic treatment regime where a lack of efficacy is detected.

In the wild population the potoroos seem to have adapted to low levels of infestations with nematodes (strongyles and nematode larvae) and protozoans (unidentified coccidian oocysts and *Eimeria*) with minimal associated clinical effects seen over the course of the study. Four wild individuals showed moderate to severe levels of infestation with nematodes but again a lack of faecal soiling or visual evidence of endoparasitic disease was seen, other than sub-optimal body condition which was present in 63.3% (19/30) of wild potoroos.

4.2.4 Conclusion

A dominance of strongyle nematode infections were found in Gilbert's potoroos, 60.5% of the population had a low level of infestation (1+) at any one point over the course of the study. The highest prevalence of infection with protozoans was of unidentified coccidian oocysts, 11.3% had mild numbers (1+) of unidentified oocysts at one point during the course of the study.

Moderate to heavy nematode infestations were only present in wild potoroos in sub-optimal body condition. All wild potoroos in sub-optimal body condition had a mild burden (1+) of strongyle eggs, however they showed no associated clinical effects, inferring endemic infestation with strongyles. Protozoal infections were only seen in mild burdens in individuals in sub-optimal body condition and did not seem to cause clinical disease.

Clinical signs of gastrointestinal disease, including loose and pungent smelling faeces and weightloss were associated with *Potostrongylus* in a wild male Gilbert's potoroo. This finding in conjunction with histopathology records reporting haemorrhagic, blood-tinged, pasty contents of the terminal small intestine, with the presence of *Potostrongylus temperatus* inciting a moderate inflammatory response, support the pathogenicity of this species in the Gilbert's potoroo.

The presence of strongyle eggs, strongyle larvae, unidentified nematode larvae and *Trichuris* sp. in the captive colony reveals current anthelmintic practices are not effective. However, there are no clinical signs of disease and all captive individuals are in good body condition. It is recommended that captive individuals are not released back into the wild population after undergoing the anthelmintic treatment regime if a lack of efficacy is detected. When considering antiparasitic treatments prior to translocation it may be prudent to first run a screening faecal flotation examination to detect current parasite burden. If a moderate or high parasite burden is detected, and given the associated stress of bringing a wild individual into captivity prior to translocation, routine parasiticidal treatment including coccidiostats should be considered and the individual quarantined for a minimum of 30 days to ensure follow up diagnostics can be undertaken to check parasite burden.

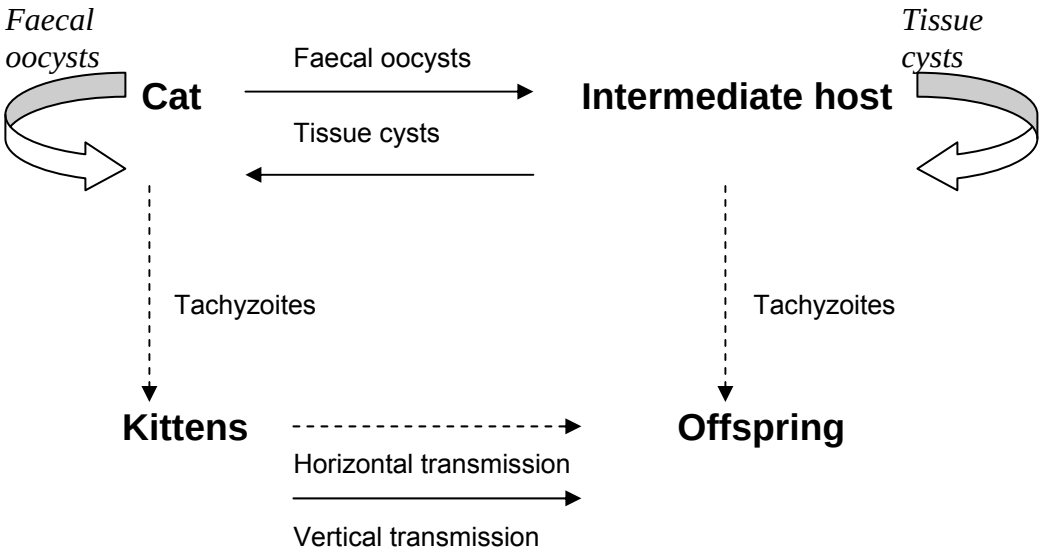
4.3 TOXOPLASMA

4.3.1 Introduction

Toxoplasmosis is a disease caused by the protozoal parasite, *Toxoplasma gondii.* It has been included as a separate sub-chapter owing to its clinical importance to marsupial medicine. *Toxoplasma* has a worldwide distribution and was probably introduced into Australia with the arrival of cats, the definitive host and main reservoir of the parasite (Johnson *et al*. 1988). A number of other mammals and birds may act as intermediate

hosts. Three infective stages of *Toxoplasma* have been identified: tachyzoites (the rapidly multiplying form), bradyzoites (the tissue cyst form), and sporozoites (found within oocysts) (Figure 4.13). Infection may be acquired via ingestion of tissue cysts in animal tissue, ingestion of faeces containing oocysts or transplacentally (Obenorf and Munday 1990, Dubey 1994).

Figure 4.13 Life cycle of *Toxoplasma gondii* (Dubey *et al*. 1995).



The cat becomes infected by ingesting meat containing tissue cysts from an animal intermediate host. These replicate within the cat's gastrointestinal tract, culminating in the release of oocysts in the faeces. It has been calculated that the number of oocysts shed in a 20g cat stool can be in the order of 2 to 20 million, and after faecal decomposition, the local soil concentration can be as high as 100,000 oocysts/g (Frenkel *et al.* 1975). Oocysts are first seen in the faeces three days post infection, but can be released up to 20 days post infection. The oocysts must be exposed to air for 24 hours before they sporulate and become infective. In soil, *Toxoplasma gondii* oocysts have been reported to remain infective for up to 18 months (Frenkel *et al.* 1975).

Certain taxonomic groups appear to have heightened susceptibility to Toxoplasmosis, in particular Australian marsupials (macropods), new world primates (squirrel monkeys) and prosimians (particularly ring-tailed lemurs). Dubey and Beattie (1988) postulate that this susceptibility may be due to the reduced evolutionary exposure of these groups to felids, as well as ecological and genetic factors. There were no cats in Australia prior to European settlement, as such Australian marsupials may not have been exposed to *Toxoplasma gondii* oocysts, and so are immunologically naïve and more susceptible to its effects. This view is strengthened by the fact that there is reportedly no clinical effect from *Toxoplasma* infection in the only non-Australian marsupial, the American opossum (*Didelphis marsupialis),* which has co-evolved in close proximity to felids (Canfield 1990). Conversely, new world monkeys, which geographically have co-evolved in close proximity to felids, are primarily tree dwellers, and therefore unlikely to come into contact with *Toxoplasma gondii* cysts due to ecological factors. Again this results in a similar immunologically naïve population that is more susceptible to the effects of disease.

Toxoplasmosis is common in Australian marsupials, and occurs as sub-clinical infection or overt disease (Munday 1978). Toxoplasmosis has been reported in wild ringtail and brushtail possums (Rose 1999), eastern-barred bandicoots (Obendorf and Munday 1990), agile wallabies, red kangaroos, eastern-grey kangaroos, red-necked wallabies, quokkas, black wallabies, and the rufous-bellied pademelon (Speare *et al*. 1989). In captivity, wombats (Arundel, Barker and Beveridge 1977, Munday 1988 and Hartley 2006) and captive koalas (Dickens 1978) have succumbed to the disease. In most cases diagnosis was based on the identification of *Toxoplasma* on histopathology, and only rarely by mouse inoculation or the detection of antibodies. An outbreak of toxoplasmosis was reported at the Knoxville Zoological Park, Tennessee, USA in 1984 in which thirteen adult marsupials; five long-nosed potoroos, three tammar wallabies, three western-grey kangaroos, and two red kangaroos, became lethargic, developed respiratory distress and died (Patton *et al.* 1986).

Infection was confirmed by macroscopic and histological findings and the results of mouse inoculation. This is the only report of a potoroo death associated with toxoplasmosis.

Marsupials are primarily infected when they ingest felid faecal matter or material contaominated by feline faeces containing sporulated oocysts, although transplacental (vertical) transmission has been reported (Rose 1999). Bettiol *et al.* (2000) suggested that eastern barred bandicoots could acquire infection through eating arthropods and in particular earthworms that had been exposed to *Toxoplasma* oocysts. Such invertebrates may acquire infection through exposure to contaminated soil, plant matter or felid faecal matter, and then act as mechanical hosts by harbouring oocysts within their gastro-intestinal tract (Frenkel *et al*. 1975).

Infection ensues when sporozoites are released from ingested oocysts, replicating in the tissues of the intestine and associated lymph tissue. Rapid replication occurs (the sporozoites are now referred to as tachyzoites) and tachyzoites disseminate through the circulatory system and into the tissue to form cysts. Cysts contain bradyzoites, and are most commonly found in the brain, liver, retina and throughout the muscle (Rose 1999). Although cysts may remain dormant for extensive periods, they can be triggered to release their bradyzoites which become tachyzoites and re-initiate active infection. Stress reducing immunocompetence is a major factor initiating this process (Attwood *et al*. 1975). Therefore clinical disease in naïve animals is caused by initial exposure to *Toxoplasma gondii* or by recrudescent infection in times of stress (Hartley 2006).

Clinical signs are primarily associated with lesions in the central nervous system, lungs and liver and can be highly variable ranging from lethargy, weakness, pyrexia, unnatural daytime activity, diarrhoea, emesis, uveitis, retinitis, cataract formation, incoordination (staggering), muscle stiffness, convulsions, coma, respiratory distress to peracute death (Attwood *et al.* 1975, Obendorf and Munday 1990, Rose 1999). Slow growth rates have been reported in some hand raised animals with *Toxoplasma gondii* infection (Rose 1999). A lack of clinical

signs with sudden death has also been recorded in macropods (Boorman *et al.* 1977), in dasyurids (Attwood *et al.* 1975), and in bandicoots (Pope *et al.* 1957). Often there are minimal lesions seen on post mortem and examination of stained organ impression smears and histopathology is required to diagnose the condition (Hartley 2006).

Serum concentrations of aspartate aminotransferase (AST), creatine kinase (CK) and alanine aminotransferase (ALT) are often elevated on standard biochemical panels in marsupials (Rose 1999).

4.3.1.1 Diagnostic testing

The detection of antibodies in the serum suggests previous or current infection with *Toxoplasma.* A number of tests are available for diagnosis of *Toxoplasma* infection in domestic animals including: methylene blue dye binding (MBD), indirect immunofluoresence antibody (IFA), indirect haemagglutination, and the enzyme-linked immunosorbent assay (ELISA). However, the majority of these are not suitable for marsupials due to their host-specificity (Garrell 1999). Therefore the direct and modified agglutination tests (DAT and MAT) are the current method of choice for *Toxoplasma* infection diagnosis in marsupials.

When exposed to *Toxoplasma* the animal will mount an immune response leading to an increase in class G immunoglobulin (IgG) over a two to three week period. This can stay elevated for the life of the individual. Class M immunoglobulin (IgM) on the other hand will rise within days of infection (Garrell 1999). It has a shorter half life than IgG and IgM antibody levels will usually revert back to normal within six to twelve weeks.

The DAT detects IgG and IgM, and used on its own lacks sensitivity and specificity (Garrell 1999). False positives are common due to non-specific IgM binding to the formalin-treated tachyzoites (Desmonts and Remington 1980). The MAT is treated to destroy the IgM so that it only detects IgG. The purpose of running the DAT and MAT tests together is to increase the sensitivity and specificity of the testing and enable diagnosis of acute versus

chronic infection from one blood sample, rather than having to check multiple blood samples for rising titres (Table 4.14).

Table 4.14 Interpretation of DAT and MAT for Toxoplasma.

MAT	DAT	Interpretation
Detects IgG - initially rises over 2-3 weeks and can stay elevated lifelong	Detects IgG and IgM	DAT-MAT=IgM - rises within days of infection and reverts to normal after 6 weeks to 3 months
	<1/64	**Negative** – often non-specific IgM
	>=1/64	**Suspicious** – early infection
>1/64	>1/64	Predominantly IgG Similar DAT and MAT – **Positive**
>1/64	>1/64 (usually a higher denominator than MAT)	Predominantly IgG (DAT usually has a higher denominator than MAT) **Positive indicates current infection**
>1/256 – 1/4096	>1/256 – 1/4096	**Infection resolved** IgG will remain positive for an extended period (from 2 yrs to lifelong).

As the MAT tests only for IgG, the difference between a DAT titre and a MAT titre from the same animal at the same time will represent the IgM concentration. Therefore, if MAT and DAT are the same (e.g. 1/1024) then it is predominantly IgG and representative of previous exposure and possible latent infection. Low titres in the DAT (1/16, 1/64) with no MAT titre have been found to be usually due to non-specific IgM in serum and samples yielding such results can therefore be classed as negative for *Toxoplasma* antibody. Normally, high levels of IgM in the serum relate to specific *Toxoplama* antibody and are indicative of current infection.

Agglutination in the MAT test at any titre corresponds to exposure to *Toxoplasma* (P. Statham, personal communication, July 2007).

Although cut off points are variable at different laboratories, generally a DAT titre <64 and no reaction in the MAT would be classified as negative. A negative result indicates one of four possibilities:

- The individual is not and never was infected.

- The infection is so recent that detectable antibody responses have not yet developed.
- The host was previously infected, but immunity was short term and antibodies are no longer present in detectable quantities.
- The host is or was infected, but was not capable of producing antibodies against infection (Scott 1988).

A DAT titre of >64, and a lack of MAT titre would be suspicious of early infection. A DAT and MAT titre of >/= 64 would reflect primarily an IgG response and the result would be positive. A positive result indicates one of four possibilities:

- Current infection.
- Previous infection to which the host is now immune.
- Cross-reaction with shared antibody from another infection.
- The presence of antibodies transferred from mother to offspring (Hartley 2006).

In the case of current infection, a positive DAT with a lack of MAT titre would be seen initially. This would then progress over time to a positive DAT and MAT (with DAT usually having a higher denominator compared to MAT).

IgG titres should typically increase by four fold, that is two dilutions or greater over a period of two to three weeks during the course of active infection (Hartley 2006). Once infection has resolved IgM should be negative, and IgG will remain positive for an extended period (usually greater than two years and may be lifelong). In such cases titres are commonly 1-256 - 1/4096.

MAT titres should also be interpreted in terms of age, as MAT titres from juvenile macropods can show false positive readings owing to passive transfer of maternal IgG (Miller 2003).

Tissue cysts have also not been detected in any animals less than six months of age (Attwood *et al.* 1975).

The use of these tests in sick marsupials may confirm a diagnosis of acute toxoplasmosis and allow commencement of appropriate therapy.

A commercially available latex agglutination test (LA) TOXOLATEX® (Fumouze Laboratoires, France) has been used in some marsupial species, including the northern quoll (*Dasyurus hallucatus)* (Oakwood and Pritchard 1999). Its ease of interpretation, rapid results (six minutes) and availability make it another viable option for remote disease investigations. Positive controls are not available for marsupial species however the TOXOLATEX® test has produced a close correlation in results (97%) to that obtained by IFA and IHA for randomly selected human sera (Robert and Senet 1984). The suitability of this test for use in marsupials warrants further investigation only if DAT and MAT testing is undertaken concurrently and the results compared and contrasted (Garrell 1999).

Histopathology and immunohistochemistry provides definitive diagnosis of infection (Hartley 2006). Typical lesions include interstitial pneumonia, (plus or minus free and intracellular tachyzoites) multifocal granulomatous encephalitis and meningitis, multifocal granulomatous, necrotizing hepatitis and granulomatous gastroenteritis, (plus or minus free and intracellular tachyzoites) (Patton *et al.* 1986). Canfield (1990) summarised clinical signs, necropsy findings and histopathological changes in 43 macropods, two wombats, eight bandicoots, two koalas, six possums, 15 dasyurids, two numbats, and one bilby with *Toxoplasma* infection. Animals either exhibited sudden death without clinical signs or showed evidence of respiratory, neurological or enteric disease. The most commonly reported gross necropsy findings included pulmonary congestion, oedema and lung consolidation, lymphadenomegaly, splenomegaly, adrenal enlargement and reddening, gastrointestinal reddening and ulceration, and pancreatic swelling. However, in many

animals lacking clinical signs or even in cases exhibiting clinical signs, minimal necropsy lesions were found. Gibb *et al.* (1966), found no gross necropsy lesions in Rottnest Island quokkas affected by toxoplasmosis. Therefore, the presence or absence of clinical signs does not always seem to correlate with gross necropsy findings, necessitating histological and immunohistochemical examination in conjunction with serological testing for definitive diagnosis.

New molecular methods are being investigated to aid in the diagnosis of toxoplasmosis. The ability to identify *Toxoplasma gondii* DNA in biological samples including body fluids and tissues provides immediate evidence of the organisms presence, in comparison to serology which is dependent on a host response (Jones *et al*. 2000). However PCR techniques lack the ability to differentiate active from chronic infection (Lee 1999) and false negatives may be obtained (similar to histopathology) if the site containing infected tissue cysts is not sampled (Guy *et al.* 1996) due to the typically focal and sparse distribution of lesions in tissues (da Silva and Langoni 2000). A one tube hemi-nested PCR technique was shown to be highly sensitive and specific in detecting *Toxoplasma gondii* DNA in the tissues obtained from Australian marsupials that were positive for *Toxoplasma gondii* via histopathology or serology or both (Adams 2003). This technique could have future widespread application due to its sensitivity and cross species reactivity, however at present it is only experimental and must be interpreted in light of clinical, histological and serological evidence.

4.3.1.2 Treatment

The disease in Australian marsupials will inevitably be fatal, and many cases will only be diagnosed post mortem (Jackson 2003). George (1990) advocated euthanasia in any severely affected animal owing to the pain associated with encephalitis. This view may be justified when assessing the potential for inducing myopathic states from daily treatments and handling of wild or captive animals not used to such levels of human interaction. Toxoplasmosis may also recrudesce and reactivate in times of stress (Booth 1999),

necessitating strict ongoing management protocols to minimize the potential for this occurring.

Treatment, is generally unrewarding. Potentiated sulphonamides, sulphadimidine and pyrimethamine or clindamycin (11mg/kg body weight twice daily orally or intra-muscularly for a minimum of 30 days) are first line treatment options. Recently, a human preparation Atovaquone (Wellvone® GlaxoSmithKline, Australia) has been trialled at 50-100mg/kg/day for 30 days. The relative expense of this drug and the poor clinical response achieved point to a need for further drug trials in marsupial species (Blyde 1999). Attempts to develop a vaccine have been universally unsuccessful (Lynch *et al*. 1993).

4.3.2 Materials and methods

Trapping, initial health assessment, anaesthesia, physical exam and blood collection of the wild and captive population was described in 4.1.2. The whole blood was centrifuged within four hours and a 0.5mL serum sample, sent to Murdoch University via overnight courier on ice. This sample was then forwarded to the Australian Animal Health Laboratory in Tasmania (AAHL) for the DAT and MAT tests which were conducted according to the following protocol.

The commercial kit Antigene Toxo-AD and microtitre plate reagents (BioMerieux SA, Marcy l'Etoile, France) were used to perform the DAT and MAT tests. Formalin-treated *T.gondii* tachyzoites were used as the antigen. In the DAT, a 25µL serum sample was initially tested at 1:16 and titrated in two-fold serial dilutions to 1:4096. In the MAT, a 25µL sample of 0.2 M-mercaptoethanol (2-ME) (BDH Poole, England) was mixed in phosphate buffered saline prior to being similarly tested in serial dilutions. The kit included positive and negative control sera BioMerieux (Toxotrol A cat. # 7 543 1). Agglutination reactions were graded as follows:

- Positive - when *Toxoplasma* organisms formed a dense layer at least covering half of the well's base,
- Partial - if agglutination covered less than half of the well's base,
- Negative - if only a small aggregrate was found at the well's base.

The results of the agglutination reaction were then expressed as the reciprocal of the highest serial dilution giving a positive reaction. Results were read using a magnified mirror microtitre plate reader.

4.3.3 Results

Minimal clinical or serological evidence of *Toxoplasma* was seen in all but two individuals (n=26). These were juvenile male GP M104 captured during the November 2005 trapping session, and adult male GP M83 captured during the June 2006 trapping session.

Male GP 104 had a 1/256 DAT titre, although the MAT was negative. This DAT titre while greater than 1/64, was most likely due to non-specific IgM (anti-*Toxoplasma* IgM antibody). Such results are usually considered by AAHL to be negative, given the negative MAT titre and the lack of specificity of the DAT test (Desmonts and Remington 1980). However, despite the lack of clinical signs it is possible this potoroo was in the very acute stages of *Toxoplasma* infection. Unfortunately this animal was not re-trapped during the study period to test this theory.

Male GP 83 had a positive 1/64 DAT titre in June 2006 and a negative MAT. However, DAT and MAT results were subsequently normal in samples collected from this individual during the March and November 2006 trapping sessions. The June 2006 results are most likely attributable to a non-specific IgM response. It is unlikely that these results would have been

associated with early stage infection given the subsequent lack of antibody in November 2006 and the fact that the individual did not display any clinical signs of toxoplasmosis.

No histological evidence of *Toxoplasma* infection was found in any tissue samples from wild or captive Gilbert's potoroos opportunistically examined during necropsy examinations (D. Forshaw personal communication, June 2007).

4.3.4 Discussion

Every captive Gilbert's potoroo that died was necropsied and histologically examined. However, in the wild the possibility of finding a body that is not too autolysed for histological exam is very low. Given the lack of serological response indicating active or latent infection, Gilbert's potoroos seem to be either un-exposed to *Toxoplasma* or the DAT and MAT serological screening test must be poorly predictive (which seems unlikely given its sensitivity and specificity in other macropods). Of the 26 Gilbert's potoroos sampled over a three year period only two serological responses (7.6%), which were probably non-specific IgM responses, were detected. Therefore, it is likely the population consists of naïve individuals highly susceptible to disease.

Australian marsupials, especially those that are immunologically vulnerable, seem highly susceptible to opportunistic pathogens such as *Toxoplasma*. Perhaps the most striking similarities to the findings in the Gilbert's potoroos are those of the eastern-barred bandicoot in Tasmania. Here, Bettiol *et al.* (2000) conducted an initial serological study of the free-ranging population and found over 95% of the population was seronegative and therefore unexposed to *Toxoplasma*. This field study prompted an experimental investigation to study the course of the disease and formation of antibodies following oral inoculation with *Toxoplasma gondii* oocysts. Two bandicoots were inoculated and both died, at 15 and 17 days post infection, from acute toxoplasmosis confirmed by an increased DAT titre (negative

MAT), clinical signs and necropsy examination. The affected bandicoots displayed interstititial pneumonia, hepatocellular necrosis and acute inflammation in association with tachyzoites, which were also found in the skeletal muscle, pancreas and muscular layers and submucosa of the small intestine. These findings indicated that eastern-barred bandicoots are highly susceptible to *Toxoplasma* and reinforced the significance of *Toxoplasma* as a potential agent that could result in population decline of the eastern-barred bandicoot.

Earlier serological studies in this species by Obendorf (1996) demonstrated 10 of 150 (6.7%) bandicoots were positive for antibodies to *Toxoplasma*. Five of these individuals were not re-trapped after initial seroconversion, and three animals demonstrated high MAT titres on the subsequent two blood sample collections (three months apart). One sero-positive bandicoot was found dead in a trap with necropsy and histological findings consistent with generalised toxoplasmosis, and the other had neurological deficits consistent with toxoplasmosis, yet was accidentally released and never recaptured. Seven of the 150 bandicoots (4.6%) initially reacted and had a low DAT titre. However, on subsequent testing these animals had negative DAT titres, never developed MAT titres and were re-classified to be antibody negative. The remaining 143 animals (95.4%) showed no antibody response and 68% of these sero-negative bandicoots were retrapped.

The inability to retrap seroconverted individuals, with raised DAT and MAT titres >64, was postulated to provide evidence that eastern barred bandicoots do not act as long standing inapparent carriers of *Toxoplasma.* However, the presence of high MAT titres in three animals provided evidence that some animals can survive the initial acute infection and seroconversion. To definitively classify Gilbert's potoroos as highly susceptibility to toxoplasmosis would require inoculation studies. These cannot be conducted due to the critically endangered nature of this species. However, by drawing parallels to other

Australian marsupials, and in particular the eastern barred bandicoot, it seems prudent to establish strategies to minimise exposure to *Toxoplasma*.

4.3.5 Conclusion

The Gilbert's potoroo seems largely unexposed to *Toxoplasma*, however with such a low prevalence of infection (7.6%) it is likely that the population consists of naïve indiviuals highly susceptible to disease. The analogous long-nosed potoroo is susceptible and given the heightened susceptibility displayed by Australian marsupials such as the eastern-barred bandicoot, it should remain a differential diagnosis in cases of sudden death and non-specific lethargy and neurological disease.

The most likely route of *Toxoplasma* transmission for captive potoroos would be via soil containing felid faecal matter being placed in their enclosures during soil changes. This is a possibility given the known feral cat population in the area. Alternatively, the ingestion of oocyst-laden invertebrates which could potentially inhabit the captive enclosures could result in infection. This is less likely with the wild population given the diet of wild potoroos is primarily 90% hypophygeal fungi. Transplacental (vertical) transmission would require a female to have a serological titre which is not currently apparent in the potoroos tested.

Any preventative strategies need to address the persistence of the parasite in the environment which is believed to be up to eighteen months (Frenkel *et al*. 1975). To minimise the risk of infection, all soil added which is added to the captive colony pens should be covered upon delivery and preferentially pre-treated, and then stored in a covered container to minimise contamination by feral cats using this sand as a litter tray. All infective forms of *Toxoplasma* are heat labile, and are destroyed by dry heat at 65ºC, boiling water, iodine and ammonia (Blyde 1999). Potential methods of decontamination include the soaking of soil in a 1 in 10 dilution of ammonia (bleach) and boiling. This is not practical on a large scale. Therefore, it may be easier to remove the top layer of soil from the collection

point (as cats do not tend to dig deeply when defaecating) and then cover and store the soil until required.

The established practice of ensuring that shoe covers are worn on entering the captive colony will also help prevent the accidental introduction of cat faeces into the captive colony. Insect control to minimise arthropod vectors should also be addressed. In the wild, reducing the risk of the potoroos contracting *Toxoplasma* relies on feral cat control.

4.4 HAEMOPARASITES

4.4.1 Introduction

A number of haemoparasites have been recorded in Australian marsupials. These include haemogregarine infections (Welsh *et al.* 1910), *Babesia* infections (Mackerras 1959), *Theileria* infections (Mackerras 1959, Lee 2004), *Haemobartonella* infections (Mackerras 1959), *Hepatozoan* infections (Mackerras 1959 and Mykytowycz 1964), and *Trypanosoma* infections (Mackerras 1959).

Detailed information on the piroplasms infecting native mammals is limited. Only seven recorded species of piroplasms infecting the following two monotreme and six marsupial species exist including: the platypus (*Ornithorhynchus anatinus)* (Mackerras 1958), short-beaked echidna (Backhouse and Bollinger 1957), brown antechinus (*Antechinus stuartii)* (Arundel *et al.* 1977), northern-brown bandicoot (*Isoodon macrourus)* (Seddon and Albiston 1966), southern-brown bandicoot (Mackerras 1959), long-nosed bandicoot (*Permales nasuta)* (Mackerras 1959), long-nosed potoroo (Mackerras 1959), and the Proserpine rock-wallaby (*Petrogale persephone) (*O'Donaghue 1997*)*. These records primarily comprise individual case reports and incidental findings during other studies (O'Donoghue 1997).

The pathological effects of haemoparasites in Australian marsupials are still under investigation. However, Clark (2004) commented that under 'normal' conditions many of the organisms do not seem to cause any clinical effect. It is only if, or when the individual becomes immunocompromised that clinical effects may become apparent, although there were no references provided to validate this comment. The most commonly reported haematological finding in association with haemoparasitism in mammals is a regenerative anaemia (Clark 2004). This is thought to result from the host incited immune response or by the physical effects of parasite reproduction on cells. Certain haematozoa such as *Hepatozoon* spp. can also cause destruction of endothelial cells or hepatocytes with associated necrosis and inflammation being reported (Clark 2004).

4.4.1.1 Babesia spp. and *Theileria* spp.

In 2003, veterinary pathologist Dr David Forshaw from DAFWA, Albany examined haemoparasites in blood smears from deceased Gilbert's potoroos (Lee 2004). These were morphologically likened to piroplasms of the genus *Babesia* or *Theileria*, later confirmed by a veterinary clinical pathologist (P. Clark, personal communication, July, 2007). Subsequent direct microscopic visualisation of 14 blood and tissue samples confirmed *Babesia/Theileria*-like intra-erythrocytic parasites. *Theileria* spp. are pleomorphic, round, ovoid, rod or irregularly shaped intra-erythrocytic and intra-lymphocytic parasites (Clark 2004). Lee (2004) molecularly characterised this observed haemoparasite in the Gilbert's potoroo and assigned the name *Theileria potorous.* At the same time, Lee (2004) also recorded the presence of a closely related organism *Theileria permalis,* in the long-nosed potoroo. High grade parasitaemias were observed in the blood of all Gilbert's potoroos infected with *Theileria potorous.* However, no corresponding clinical disease was detected. The clinical signs of Theileriosis are highly variable in animals, ranging from undetectable, to moderate illness, through to acute and fatal disease (Barnett 1977). Clinically this may be evident as pyrexia, anorexia, and weight loss. Gastro-intestinal signs include diarrhoea and upper respiratory signs of conjunctivitis, nasal discharge and coughing. Petechial haemorrhage of

serous and mucous membranes may be seen in severe cases and terminally, pulmonary oedema and respiratory distress is observed (Barnett 1977). The clinical presentation of the disease is dependent on factors including host susceptibility, virulence between species and strain, and the number of vectors, to which hosts are exposed (Barnett 1977).

Given the observed high parasitaemia, Lee (2004) proposed an acquired tolerance to *Theileria* in the Gilbert's potoroo. It was commented that piroplasms and marsupials may have evolved together over a long period leading to the observed lack of host response (Lee 2004). This is exemplified by *Theileria ornithorhynchi* found commonly in the platypus (Mackerras 1959). This species of *Theileria* is usually regarded to be non-pathogenic and has been reported to typically affect less than 1% of erythrocytes and leukocytes. However in one report, a juvenile was found to have 12% of its erythrocytes infected (Munday *et al*. 1988) and had an associated haemolytic anaemia. This example confirms Barnett's (1977) statement that the clinical presentation of haemoparasitic disease in marsupials is dependent on a number of host factors, and in this case, the number of vectors. The increased percentage of infected erythrocytes in this individual possibly led to an overwhelming host response to the parasite, resulting in disease. Another example of the way hosts can be affected by the presence of haemoparasities is demonstrated by the findings of Mackerras (1959) in relation to a southern brown bandicoot which was found to have red cells infected with *Theileria permalis* with an associated poikilocytocis, yet there were no indications of illness.

Reports do however exist of pathogenic haemoparasites in marsupials. *Babesia* was found in blood smears of moribund and recently dead, brown antechinus (*Antechinus stuartii),* (Arundel *et al.* 1977). *Babesia* species are relatively large, round to pyriform or irregular intra-erythrocytic organisms (Clark 2004). Necropsy of the antechinus showed splenic lymphoid follicles, gastro-intestinal ulceration, haemorrhage and haemoglobinuria. It was postulated that the *Babesia* had caused a haemolytic anaemia (Arundel *et al.* 1977, Barker

et al. 1978) and was transmitted by the tick vector, *Ixodes antechinii* owing to the widespread infestation with ticks in this species and the particular individual. However, no clinical effects of *Babesia* have been reported in other native mammals, including the platypus (Mackerras 1958), short-beaked echidna (Backhouse and Bollinger 1957), northern brown bandicoot (Seddon and Albiston 1966), long-nosed bandicoot (Mackerras 1959), long-nosed potoroo (Mackerras 1959), and the Proserpine rock-wallaby (O'Donaghue 1997).

Recently molecular technologies have extended to haematozoa identification and appear far more sensitive than conventional methods of visualization such as the preparation of thin blood smears. Using conventional methods, an infection of greater than 0.1% of erythrocytes will be visible using haematological stains such as Wright's and Giemsa (Gaunt 2000). Jefferies *et al.* (2003) however, has developed a PCR for *Babesia* spp. whereby a 0.000003% parasitaemia is detectable. Unfortunately this technology is still research-based and not widely available. As such, most identification is reliant on direct visualisation. A common pitfall using conventional techniques is contamination from sources such as dust and sand. These sources must be minimised as otherwise an accurate diagnosis may not be achieved (Clark 2004).

4.4.1.2 Trypanosomes

Recently trypanosomes have been found in Australian marsupials and monotremes, including the eastern-grey kangaroo, wombat, platypus (Noyes *et al.* 2000) and the swamp wallaby (Hamilton 2005). Trypanosomes are extracellular, flagellated protozoal organisms (Clark 2004), typically transmitted by blood sucking arthropod or aquatic leech vectors in domestic animals. Little data exists on modes of transmission in Australian wildlife species (Hamilton *et al*. 2005). The presence of trypanosomes in a blood sucking invertebrate does not establish vector status, as the presence of trypanosomes in such invertebrates may only be associated with a blood meal from an infected host. However, trypanosomes have been

found in tick nymphs (*Ixodes holocyclus*) removed from bandicoots infected with *Trypanosoma thylacis* (Mackerras 1959). The platypus tick, *Ixodes ornithorhynchi*, blood sucking flies and aquatic leeches have all been suggested as possible vectors of the platypus trypanosome, *Trypanosoma binneyi* (Mackerras 1959). A trypanosome has also recently been identified in Gilbert's potoroos (J. Meinema personal communication, July 2007).

As is the case with diagnosis of haematozoa, the use of molecular techniques such as PCR for the detection of trypanosomes has advantages, including sensitivity and rapidity of detection, when compared to conventional techniques (Noyes *et al.* 1999). Trypanosomes are usually only found in low numbers and therefore not commonly visible on direct examination of thin blood smears. Blood samples to be screened by conventional methods are typically placed into a haematocrit centrifuge to concentrate the parasites within the buffy coat layer (Woo 1969). The buffy coat is then smeared and stained onto a slide for identification (Clark 2004). Alternatively, culture can be used to detect trypanosomes. This method is time consuming and as such is not commonly used for screening wildlife populations, although it is more sensitive than the haematocrit concentration method (Noyes *et al.* 1999).

4.4.1.3 Microfilariae

Microfilariae of filaroid nematodes belonging to the *Onchocercidae* have also been described in Australian mammals (Mackerras 1962). The microfilariae are the larvae of the adults and appear on blood films to be slender bodies, lacking internal differentiation (Clark 2004). Typical sites of colonisation include body cavities, lungs, blood or lymphatic vessels, joints, connective tissue and intermuscular regions (Clark 2004).

The life cycle involves ingestion by haemophagous arthropods, and larval development in tissues of an intermediate host. These larvae are then passed onto a definitive host through another arthropod blood sucking vector, and the larvae develop into adults.

Filaroid nematodes from genera including *Pelecitus, Breinlia, Cercopithifilaria* and *Sprattia* have all been identified from Australian marsupials (Clark 2004). Of particular interest is the *Breinlia* spp. and *Breinlia macropi* which were isolated from the peritoneal and pleural cavities of an aged female Gilbert's potoroo found dead, assumed to have been recently regurgitated by a python, described in Section 4.2.1.7.

Breinlia spp., also known as *Dipetalonema* has been identified in a number of marsupial species including, the long-nosed potoroo, rufous bettong, agile wallaby, antelopine kangaroo *(Macropus antilopinus),* black-striped wallaby *(Macropus dorsalis),* eastern grey kangaroo, black-gloved wallaby *(Macropus irma),* Parry's wallaby *(Macropus parryi),* Euro or common wallaroo *(Macropus robustus),* red-necked wallaby, red kangaroo, bridled nail-tail wallaby, northern nail-tail wallaby, short-eared rock wallaby *(Petrogale brachyotis),* pygmy rock wallaby *(Petrogale concinna),* Herbert's rock wallaby *(Petrogale herberti*), unadorned rock wallaby *(Petrogale inornata),* brush-tailed rock wallaby, quokka, Tasmanian pademelon *(Thylogale billardierii),* red legged pademelon *(Thylogale stigmatica),* and the swamp wallaby *(*Mackerras 1962, Spratt and Varughese 1975, Spratt *et al.* 1991).

Breinlia spp. typically infect host body cavities. Their microfilaria may be found in the blood and they can be identified by their unsheathed, long tapering tails (Clark 2004). Pathogenic effects from the presence of the parasite include surrounding inflammation, as was observed in a swamp wallaby (Beveridge *et al.* 1985). In this case, the presence of the microfilaria caused eosinophilia in peripheral blood, an associated microfilaraemia and a focal granulomatous splenitis as a result of sequestered microfilaria.

Microfilaria are typically diagnosed through examination of the 'leading edge' of a freshly prepared blood film. However, if low numbers are present, then concentration techniques such as the Wylie method or Modified Knott's method may aid in diagnosis (Kelly 1977).

4.4.2 Materials and methods

Trapping, initial health assessment, anaesthesia, physical examination and blood collection of the wild and captive population was described in 4.1.2. Two blood smears were also made using the cover slip method and were sent to Murdoch University for haemoparasite identification. Slides were stained using a modified Wright's stain with an automated stainer (Hematek, Bayer) using the manufacturer's instructions.

4.4.3 Results

Red cell inclusions were seen in captive potoroos GP M46, GP F57 and GP F93 in June 2006, November 2006 and March 2007. There was a high degree of parasitaemia with at least two red cells affected per 100x oil immersion view. Morphologically the inclusions were described as pleomorphic, round, or irregularly shaped intra-erythrocytic parasites, approximately 1- 2µm in length. These inclusions were thought to be *Theileria.* They were distinguished by their size, the spherical ring surrounding the bodies, their presence in lymphocytes and the smaller amount of nuclear material compared to the piroplasm, *Babesia.* No evidence of anaemia, red cell morphology changes or inflammation as evidenced by a leukocytosis in peripheral blood was found.

In June 2006, wild GP M68 and GP M116 had a similar high level of parisitaemia with *Theileria.* In November 2006 high parasitaemia with *Theileria* (Figure 4.15) was again noted with a corresponding microfilaria infection in both individuals (Figure 4.16). Again there was no evidence of anaemia, red cell morphology changes or inflammation as evidenced by a leukocytosis in peripheral blood.

Figure 4.15 Peripheral blood smear of a Gilbert's potoroo showing the presence of (a) a Howell-Jolly body and (b) intra-erythrocytic *Theileria*. The piroplasms were round, ovoid or pear shaped with 1-2 parasites present per cell. Haemoxylin and Eosin 100x.

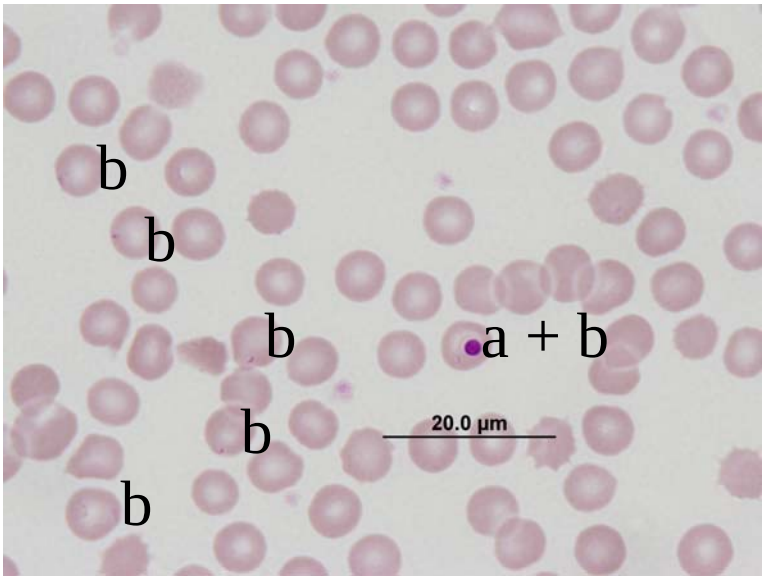
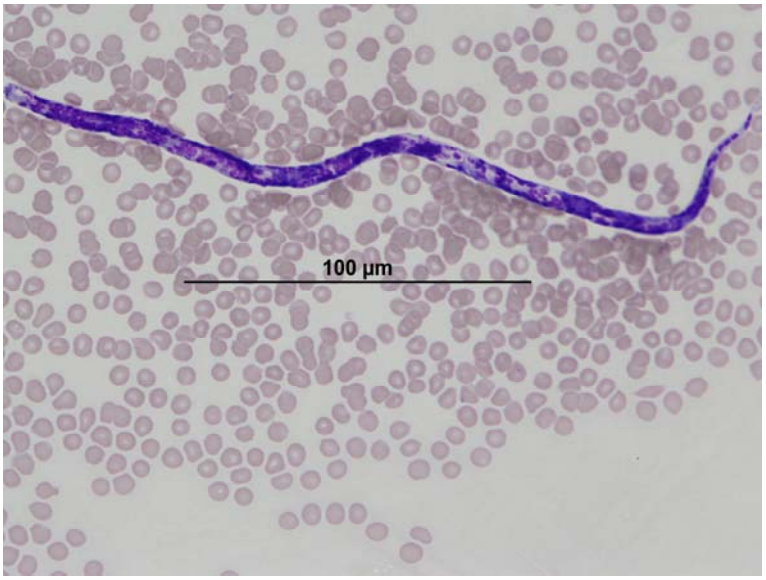


Figure 4.16 Microfilaroid in a peripheral blood smear of a Gilbert's potoroo. Haemoxylin and Eosin 40x.



4.4.4 Discussion

The piroplasm, *Theileria* reported previously in Gilbert's potoroos (Lee 2004) was found in three of nine captive potoroos over three sequential trapping sessions spanning ten months. Two of 26 wild males were also found to have a high parasitaemia in June 2006, one of which remained persistently infected for the following five months. This individual was not re-trapped in March 2007, as such no further disease screening for haemoparasites was undertaken.

In both the wild and captive population, the presence of *Theileria* was not associated with a typically regenerative anaemia, characterized by increased polychromasia of erythrocytes, reticulocytosis, macrocytosis, metarubricytosis, increased numbers of Howell-Jolly bodies, basophilic stippling or inflammation as evidenced by elevated numbers of white blood cells in peripheral blood (Clark 2004). The low numbers of Howell-Jolly bodies combined with the knowledge that Howell-Jolly bodies have been reported in clinically healthy individuals from numerous marsupial species (Clark 2004) supported the findings of Lee (2004) which did not indicate pathological consequences from the presence of *Theileria* in the Gilbert's potoroo The observed high parasitaemia possibly reflected an acquired tolerance to *Theileria* in the Gilbert's potoroo (Lee 2004). This mechanism seems similar to the minimal host effect from *Theileria ornithorhynchi* found commonly in the platypus (Mackerras 1959). Suggesting that certain species of piroplasms and marsupials may have evolved together with minimal associated pathological effect (Lee 2004).

One wild male individual in sub-optimal body condition score 2 had evidence of a low level of infection with a single microfilaria found on peripheral blood smear. This haemoparasite was identified to be *Breinlia* spp. due to its long unsheathed tapering tail (Clark 2004). No inflammation as evidenced by a leukocytosis in peripheral blood was found. Given the prior reported presence of *Breinlia* spp. and *Breinlia macropi* in Australian marsupials, including

the long-nosed and Gilbert's potoroo, the pathological consequences of the presence of this haemoparasite warrant further discussion. *Breinlia macropi* was isolated from the peritoneal and pleural cavities of an aged female Gilbert's potoroo found dead assumed to have been recently regurgitated by a python. The presence of *Breinlia macropi* microfilaria in a swamp wallaby caused a peripheral eosinophilia, and an associated microfilaraemia (Beveridge *et al.* 1985), while in many other marsupials reports are based on incidental findings of sub-clinical infection with this parasite. Given the low levels of microfilaria seen, the lack of associated eosinophilic infiltrate and the fact that the regurgitated potoroo seemed to be suffering from many concomitant diseases, it is difficult to assign significance to the presence of microfliaria in this individual. However, blood smears should continue to be evaluated for the presence of microfilaria to allow a more thorough assessment of its significance to be made.

4.4.5 Conclusion

Three of nine members of the captive colony and two of 26 members of the wild population were found to have a high parasitaemia with *Theileria*. There was no evidence of anaemia, red cell morphology changes or inflammation as evidenced by a leukocytosis in peripheral blood. While the true incidence of *Theileria* in Australian marsupials and their significance is still under investigation, results from this study, support those reported by Lee (2004) and do not indicate pathological consequences from the presence of this haemoparasite.

One wild individual in sub-optimal body condition 2 had evidence of low level infection with microfilariae on blood smear. No inflammation as evidenced by a leukocytosis in peripheral blood was found. The pathogenicity of *Breinlia* spp. remains questionable due to the low prevalence of infection, the lack of host clinical response and the minimal pathogenicity that has been reported for this species in other Australian marsupials.

It is recommended that haemoparasite screening via blood smear should continue to be undertaken as part of health screening any potoroo to be translocated or any potoroo undergoing health evaluation for diagnostic purposes.

CHAPTER 5

Microbiology of the cloaca in the Gilbert’s potoroo

5.1 INTRODUCTION

To understand the potential pathogenicity of cloacal microbial flora a thorough understanding of commensal flora is required. Commensal microflora refer to micro-organisms present on body surfaces covered by epithelial cells which are exposed to the external environment, this includes the gastrointestinal and upper respiratory tract, lower urinary tract, vagina and skin (Tlaskalova-Hogenova 2004). Commensal bacteria are thought to have co-evolved with their hosts, however under certain conditions they may overcome protective host responses and exert pathology. Commensal bacteria at these various sites may be a range of obligate anaerobic, aerobic and facultative anerobic bacteria.

5.1.1 Commensal microbial flora in mammals

There are few references to commensal cloacal microbial flora in marsupials and monotremes, other than in the koala, echidna and platypus (Osawa *et al*. 1993, Whittington 1988). Furthermore, veterinary microbiology texts tend to refer to domestic mammals which possess a defined rectum and urogenital orifice as compared to the marsupial cloaca. The cloaca is the common terminal opening for rectum, urinary ducts and genital ducts in both sexes of marsupials (Johnson-Delaney 1996). This anatomical difference should be remembered when comparing microbial flora in species without a cloaca due to the potential for contamination of samples swabbed from either the urogenital orifice of the cloaca or, the rectal orifice of the cloaca.

In domestic mammals the role played by commensal urogenital flora is often uncertain (Steinhoff 2005). In females the urogenital flora is generally thought to be protective, and pathogenic strains are usually excluded, however there are some microflora species that can be pathogenic if the orifice becomes compromised. Examples of such microflora include *Streptococcus zooepidemicus* in mares with endometritis, *Escherichia coli* in a contaminated canine pyometra and the combination of *Corynebacterium pyogenes* and *Fusobacterium necrophorum* in cows with pyometra (Hirsh and Wiger 1977). If the normal flora is disturbed such as during antibiotic

treatment, the urogenital tract will often be repopulated with other, often resistant bacterial strains or species and, or yeasts and this may lead to infection if the underlying problem remains uncorrected.

Long-term studies of the aerobic flora of the genital tract in healthy breeding bitches and studs report a predominance of common opportunistic pathogens, including *Pasteurella multocida*, beta-haemolytic *Streptococcus* and *E. coli* (Bjurstrom and Linde-Forsberg 1992). These bacteria were reportedly often transferred between the dog and bitch at mating and caused no clinical effects unless the dogs had clinical signs of genital disease prior to mating (Allen and Dagnell 1982, Bjurstrom and Linde-Forsberg 1992). The female's flora also altered significantly during the reproductive cycle. *Pasteurella multocida* and beta-haemolytic *Streptococcus* were isolated more frequently during pro-estrous, oestrous, metaoestrous, pregnancy and the postpartum period, compared to *Staphylococcus intermedius,* which was only found following parturition (Bjurstrom and Linde-Forsberg 1992). No studies on anaerobic bacteria were conducted.

5.1.2 Commensal microbial flora and digestive strategy

Gastro-intestinal anatomy and digestive strategy can also alter the commensal bacterial balance found in certain sites. If gastro-intestinal sites are primarily digestive in function, as compared to fermentive or acting as a storage organ, differing bacterial populations will be found due to variations in volatile fatty acid production. Marsupials are either primary foregut or hindgut fermenters. *Phascolarctos*, as well as the families *Phalangeridae* and *Pseudocheiridae* are arboreal hindgut fermenters. Foregut fermenters include the *Macropodidae* (Hume 1999). In the hindgut fermenting *Phascolarctos*, the end products of fermentation are short chain fatty acids which increase in concentration in the caecum and proximal colon, whereas in the foregut fermenting *Macropodidae,* the stomach has the highest concentration of short chain fatty acids (Hume 1999). Foregut fermenters have several advantages over hindgut fermenters. The most important advantage is that microbial cells are synthesized in the stomach and digested in the

small intestine, where as in hindgut fermentation, synthesized microbial cells are lost in faeces (Hume 1999).

The *Macropodidae* have a large tubiform forestomach, where large amounts of digesta are retained for moderate periods of time (Hume 1978). In comparison, the hindgut fermenting *Phascolarctus* have a relatively small and simple stomach, with a correspondingly short small intestine (Hume 1999). The caecum and proximal colon, the primary sites of microbial fermentation and digesta retention, constitute 23% of the length of the total intestine and 35% of the total volume (Hume 1999). The caecum contains an adherent layer of gram-positive and gram-negative bacilli, cocci, and *Actinomycete*-like organisms, arranged along the mucosa. An *Enterobacter* sp. that degrades tannin-protein complexes found in *Eucalyptus* is also present (Osawa *et al*. 1993). The ratio of anaerobic to aerobic bacteria in the caecum is 1150:1. The caecum and proximal colon have pHs of 6.5 and 6.6 respectively, which provide favorable conditions for bacterial growth and fermentation (Hume 1999).

Potoroids have been assumed to have a similar foregut digestive strategy to macropods, with long retention times in the forestomach (Hume and Carlisle 1985). However, research by Frappel and Rose (1985) reported the long-nosed potoroo has a voluminous sacciform forestomach, and a well developed hindgut with the proximal colon and caecum having a volume of approximately 1/3 of the size of the sacciform forestomach. Carr (1970) demonstrated levels of short chain fatty acids in the forestomach, caecum and colon of the long-nosed potoroo that suggested microbial fermentation occurred in all three sites (Barnett and Reid 1961).

The long-nosed potoroo primarily feeds on a diet of fungi, seeds, plants and insects. Fungi as a food source are thought to be deficient and unbalanced in amino acids. It was assumed that the long-nosed potoroo's forestomach primarily digested fungi. Although all solid digesta entered the sacciform forestomach, due to the short retention time, only the fungi were utilised. All remaining digesta passed through to the caecum and proximal colon where slower microbial fermentation of

the fibre rich dietary components occurred (Frappel and Rose 1985). A similar situation with long retention time in the hindgut occurs in the tammar wallaby (Richardson and Wyburn 1980). Frappel and Rose's study (1985) proposed that the potoroid forestomach maintained a microbial population to correct the amino acid deficiency of the fungal diet, allowing pre-gastric fermentation, to enable utilization of usually nutritionally incomplete food sources (e.g. fungi). However, compared to the detailed studies conducted in koalas, no qualitative assessment of the bacterial population was made.

5.1.3 Specific bacteria causing gastro-intestinal and urogenital disease in marsupials and domestic animals

Literature on marsupial cloacal microbial populations and agents causing clinical disease is limited, therefore a review of common pathogens found in the gastro-intestinal and urogenital tract of domestic mammals, and also humans will be included for comparative purposes.

Of all the Australian marsupials and monotremes only two species: the echidna and platypus have reported faecal or intestinal microbial culture results. Whittington (1988) reported that of 14 captive echidnas studied, 71.4% of samples (10/14) had a population of mixed haemolytic and non-haemolytic coliforms, *Proteus* sp. was isolated from 50% of samples (7/14), *Enterococcus* sp. from 42.8% of samples (6/14), *Streptococcus* sp. from 14.3% of samples (2/14), *Salmonella typhimurium* from 14.3% of samples (2/12), and *Salmonella dublin* from 8.3% of samples (1/12). Only the *Salmonella* isolates were thought to be clinically significant. In captive platypus the following bacteria were recovered from 18 faecal, cloacal or intestinal cultures: mixed coliforms from 55.6% of samples (10/18). *Proteus* sp. from 44.4% of samples (8/18), *Aeromonas hydrophila* from 27.7% of samples (5/18), *Enterococci* from 22.2% of samples (4/18), *Salmonella typhimurium* from 11.1% of samples (2/18), *Streptococcus* sp. from 5.5% of samples (1/18), *Serratia* sp. from 5.5% of samples (1/18) and *Pseudomonas* sp. from 5.5% of samples (1/18). Again only the *Salmonella* sp. was thought to be clinically significant.

A review of the marsupial literature and important microbes causing clinical disease in the gastro-intestinal and urogenital tract of domestic mammals and humans follows.

5.1.3.1 Actinobacillus sp.

Actinobacillus are Gram-negative pleomorphic bacilli and coccobacilli, approximately 0.5µm wide and overlapping phenotypically with the *Pasteurella* group (Biberstein and Zee 1990). *Actinobacillus seminis*, *Brucella ovis* and a variety of other *Actinobacillus*-like species including *Histophilus somni (Histophilus ovis)* have been primarily associated with epididymitis in sheep (Jansen 1980). Lesions have varied from acute to chronic, uni or bi-lateral epididymitis or epididymo-orchitis (Al-Katib and Dennis 2007). Other systemic signs include purulent polyarthritis, posthitis in lambs and placentitis and abortion in ewes (Van Tonder 1973). *Actinobacillus* sp. is thought to be a commensal inhabitant of the ovine prepuce and is only involved in epididymitis, orchitis and polyarthritis in lambs when the host's integrity is compromised, for example through trauma, immaturity or stress (Biberstein and Zee 1990). In ruminants, outbreaks have been linked to the provision of rough feed damaging mucous membranes promoting invasion and infection. In horses, *Actinobacillus-suis* like organisms occur in the respiratory and genital tracts on mucous membranes (Biberstein and Zee 1990). Treatment involves antimicrobial therapy including tetracycline and chloramphenicol.

5.1.3.2 Aeromonas sp.

Aeromonas is a Gram-negative, facultative anaerobic rod that morphologically resembles members of the family *Enterobacteriaceae* (Biberstein and Zee 1990). A mixed bacterial population with predominance of *Aeromonas* sp. was reportedly the cause of non-specific enteritis, mesenteric abscessation and acute peritonitis in three wild and one captive echidna (McOrist and Smales 1986).

5.1.3.3 Bacillus sp.

Bacillus sp. are spore-forming, aerobic, Gram-positive rods, typically inhabiting soil and water (Biberstein and Zee 1990). *Bacillus anthracis,* the agent of anthrax was reported in two captive

kangaroos (Blair 1924), and these infections were fatal. *Bacillus anthracis* has been reported to infect ruminants, horses, swine, carnivores and humans. In ruminants sudden death may occur, alternatively, following incubation animals may present with fever, chills, agalactia, abortion, congested mucous membranes, haematuria, haemorrhagic diarrhea and regional oedema, mostly leading to septicaemia and death (Biberstein and Zee 1990).

Bacillus cereus can cause opportunistic infections in cattle and humans. In cattle, infection has been associated with abortion and mastitis (Logan 1988).

Bacillus piliformis, the agent of Tyzzer's disease causes an acute fatal diarrhoeal disease due to focal liver necrosis. Clinical disease has been reported in laboratory mice, rabbits, hares, gerbils, rats, hamsters, muskrats, dogs, cats, snow leopards, foals and rhesus monkeys (Biberstein and Zee 1990).

Bacillus spp. has also been isolated from necrobacillosis lesions in macropods (Beveridge 1934). Experimental inoculation of *Bacillus* (*Sphaerophorus necrophorus*) into kangaroos produced 8cm oral lesions in 16 days with resultant emaciation. The significance of *B. necrophorus* in naturally occurring lesions however, is unknown.

5.1.3.4 Bifidobacterium sp.

Bifidobacteria are non-spore forming, non-motile, Gram-positive and catalase-negative anaerobes (Biberstein and Zee 1990). *Bifidobacterium* sp. are found in humans, animals, wastewater and fermented milk (Gomes and Malcata 1999). *Bifidobacteria* are found in the mouth and the intestinal tract of humans and animals. In the adult human colon, *Bifidobacterium* sp. is one of the most abundant probiotic organisms that populate the colon (Finegold *et al.* 1983). Many probiotic nutritional supplements contain a high proportion of *Bifidobacterium* sp. used to aid restoration of commensal intestinal microflora. Certain species such as *B. longum* 536, which

is added to certain yoghurts, has been found to improve intestinal health by reducing the population of *Clostridium* sp. and the level of putrefactive substances (Ogata *et al*.1999).

5.1.3.5 Clostridium sp.

Clostridia are Gram-positive, motile, anaerobic rods affecting humans, domestic animals and avian species (Biberstein 1990). *Clostridium botulinum,* the agent of botulism, and *Clostridium tetani*, the agent of tetanus, are probably the best known non-invasive species. Other species including *Clostridium perfringens* can occur in the intestinal tract of humans and animals and in most soils, and result in wound infections and enterotoxaemia in calves, lambs, piglets and foals (Borriello 1985). Clostridia can be commensal intestinal residents in normal animals, especially adults, however during outbreaks of disease, pathogenic strains survive in the soil to infect other individuals. The bacteria can enter the host either through ingestion or wound infection. Enterotoxic disease development is dependent on the intestinal environment, which is influenced by diet and age. Over-eating, especially of protein and energy rich food, provides the perfect medium for toxigenesis (Nillo 1980) and also slows intestinal motility to favour bacterial retention and toxin absorption. Demonstration of the toxin in the contents of the small intestine is diagnostic. Treatment varies depending on the strain of bacteria.

5.1.3.6 Corynebacterium sp.

Corynebacterium are pleomorphic, non-spore forming, non-motile, aerobic Gram-positive bacilli (Biberstein and Zee 1990). *Corynebacterium* sp. *w*as isolated from the stomach of a juvenile hand-reared echidna, which died due to gastric dilation. Gastritis was evident histologically in this case (Whittington 1998).

Corynebacterium renale colonizes the urogenital tract of ruminants. Opportunistic infections of this commensal organism include bovine pyelonephritis and ovine posthitis ‘pizzle rot’ (Yangawa and Honda 1978). Occasionally it has also been implicated in urinary tract disease of horses, dogs and non-human primates (Biberstein and Zee 1990). In cattle, disease with this agent causes a cystitis proceeding to ureteritis and pyelonephritis. Chronic infections lead to bladder

wall thickening, debilitation and possible death from uremia (Yeruham *et al.* 1999). Bovine pyelonephritis is typically found in cows nearing parturition. In sheep and goats, disease with this agent causes a necrotizing inflammation of the prepuce and adjacent tissues (Biberstein 1990). *Corynebacterium renale* is urealytic so when urine is exposed to this bacterium, the urea is broken down to ammonia, and this is thought to initiate the inflammatory process. *Corynebacterium pilosum* has also been found in association with *Corynebacterium renale* (Yangawa and Honda 1978). Four of 25 Texel rams in Morayshire, Scotland were found to have died due to urethral obstruction secondary to posthitis. A profuse purulent preputial discharge was evident from which *Corynebacterium pilosum* and a *Corynebacterium renale*-like organism were identified (Scottish Agricultural College 2005, unpublished report). 'Pizzle rot' typically occurs in animals on a high protein diet (legume rich diet), which increases urea excretion and oestrogens and can cause preputial swelling and urine retention in the sheath (Biberstein and Zee 1990). *Corynebacterium renale* is susceptible to penicillin in the early stages of infection, however for most ovine cases surgical care of lesions, local antiseptic applications and dietary restriction are the mainstays of treatment (Yangawa and Honda 1978).

5.1.3.7 Lumpy Jaw or Necrobacillosis - Fusobacterium necrophorum, Arcanobacterium pyogenes and Dichelobacter nodusus

This is a disease of high importance in captive macropods (Samuel 1983), however it has also been reported to sporadically affect wild populations of kangaroos and wallabies dating back to the fossil record (Horton and Samuel 1978). Causative agents include *Fusobacterium necrophorum*, *Arcanobacterium pyogenes* and *Dichelobacter nodusus* (Blyde 1999). *Arcanobacterium pyogenes* was formally known as *Actinomyces (Corynebacterium) pyogenes*. *Fusobacterium* sp. and *Bacteroides* sp. are non-spore forming Gram-negative obligate anaerobes (Biberstein and Zee 1990). These bacteria can combine synergistically or occur singularly to cause granulomatous, suppurative lesions to the oral cavity and mandible resulting in the decay of bone and teeth loss. Clinical signs include facial swelling, weight loss, excessive salivation and tongue flicking. Dysphagia, dyspnoea, rhinitis, dull eyes, poor coat, progressive weakness

and loss of condition owing to difficulties in prehension and mastication have also been reported (Blyde 1999, Booth 1999). Other less commonly involved sites include the feet, tail, lungs, gastric mucosa and liver (Butler and Burton 1980): terminal septicaemia can also occur (Arundel *et al.* 1977).

Lumpy jaw has been associated with faecal contamination of food due to poor hygiene, overcrowding leading to stress and increased ground bacterial counts, and a diet primarily composed of soft foods and little roughage, which weakens the strength and bone density of the oral cavity making it more susceptible to abrasions. These factors combine, leading the gums to become soft, and allow bacterial invasion (Finnie 1978); cellulitis and necrosis of the tissues of the mouth then ensues (Hume *et al*. 1989). In the wild population, sporadic outbreaks were thought to be associated with drought (Tomlinson and Gooding 1954).

Species reported to have been affected by Lumpy jaw include the red kangaroo (Hamerton 1933), hill wallaroo (*Macropus robustus)* (Hamerton 1929), Parry's wallaby (Hamerton 1935), red-necked wallaby (Scott 1927), eastern-grey kangaroo (Fox 1923), tammar wallaby (Scott 1926), swamp wallaby (Rewell 1948, Arundel *et al.* 1977) and the black-tree kangaroo (*Dendrogalus ursinus)* (Fox 1938). A variable susceptibility is seen among the macropods. The red-necked wallaby, Parry's wallaby and the red kangaroo are highly susceptible, while the other species mentioned seem less susceptible (Calaby and Poole 1971).

Diagnosis is made by clinical signs and anaerobic culture of any oral lesions. Radiography to ascertain any bony involvement should also be undertaken (Booth 1999). Once clinical signs become apparent affected animals may succumb within four days to three weeks (Tucker and Millar 1953). If lesions are advanced then euthanasia should be considered. In captivity, as high numbers of animals are often kept in enclosures, there is a greater chance for cross-transfer owing to the high bacterial counts often associated with disease. Blyde (1999) recommends that if the disease is diagnosed early and the individual is highly valuable to the breeding population

then treatment should be commenced with a parenteral antibiotic combination such as amoxicillin and clavulinic acid, gentamicin and metronidazole in conjunction with the removal of necrotic teeth. Oral medication should be avoided due to the potential for these drugs to cause a dysbacteriosis of commensal bacteria, required for fermentation of food (Booth 1999). If a multi-species enclosure becomes contaminated it is recommended to spell the enclosure for three to four weeks, and move the contaminated individuals to another area while undergoing treatment to minimise the spread of bacteria (Butler 1981).

Prevention focuses on the provision of hard foods, to strengthen the teeth and gums. Foods offered however should not be sharp, as this can potentially cause gum abrasions facilitating bacterial passage. Strict hygiene is vital, all faecal matter should be removed, and feed trays placed above the ground to reduce the potential for faecal contamination when feeding. Additionally, stocking rates should not exceed standard requirements for the species as specified in individual husbandry manuals. While a commercial sheep vaccine Footvax® (Coopers Animal Health, Baulkham Hills, NSW, Australia) exists, there is debate about the efficacy of this vaccine in the prevention of lumpy jaw in macropods (Blandon *et al.* 1987, Blyde 1994).

5.1.3.8 Enterobacter sp.

Enterobacter species are opportunistic facultative pathogens, similar to other members of the family Enterobacteriaceae including *Klebsiella*, *Escherichia*, *Citrobacter*, *Proteus, Serratia*, *Salmonella*, *Shigella* species, and many others (Fraser 2007). In humans, Enterobacteriaceae are the most frequent bacterial isolates recovered from faecal samples, particularly *Enterobacter cloacae* and *Enterobacter aerogenes.* These are important nosocomial pathogens responsible for various infections, including bacteremia, lower respiratory tract infections, skin and soft tissue infections, urinary tract infections (UTIs), endocarditis, intra-abdominal infections, septic arthritis, osteomyelitis, and ophthalmic infections (Fraser 2007). In animals, *Enterobacter cloacae* and *Enterobacter aerogenes* have been frequently associated with bovine mastitis (Carter and Wise

2004). Culture of the organism and antimicrobial sensitivity is required for treatment as resistance is high (Foster and Rhoney 2005).

5.1.3.9 Escherichia sp.

The genus *Escherichia* is composed of many species, however, facultative, Gram-negative beta-haemolytic *E. coli* is by far the most important pathogen to animals (Biberstein and Zee 1990). This species typically found as a commensal in the gastrointestinal tract is an opportunistic invader. Beta-haemolytic *E. coli* has been associated with septicaemia in calves, piglets and lambs, enterotoxigenic diarrhea in newborn domestic animals and in oedema disease in piglets (Oberman 1987). Fowl can develop colibacillosis from invasive strains of *E. coli* causing embryonic death, respiratory and gastrointestinal signs or septicaemia. Opportunistic infections include urinary tract disease, pneumonia and abscessation in almost all animal species (Biberstein and Zee 1990). Treatment involves correcting fluid and electrolyte imbalances. Antimicrobial agents are required for the treatment of invasive strains of *E. coli* (Oberman 1987).

5.1.3.10 Eubacterium sp.

Eubacterium are anaerobic Gram-positive bacilli. In sows, *Eubacterium suis* has been associated with urinary tract infection. Ascending infection by the urealytic agent caused symptoms similar to bovine pyelonephritis and was particularly prevalent during pregnancy and parturition (Biberstein and Zee 1990). Treatment is usually unsuccessful.

5.1.3.11 Klebsiella sp.

Klebsiella species are common microbes comprising between 30% to 40% of the saprophytic bacterial population of the gut of wild and domestic animals (Le Minor and Veron 1982). *Klebsiella* species, in particular *Klebsiella pneumoniae*, is an opportunistic pathogen in the genitals of the mare, the udder of milking cows and in the urinary tract of the bitch (Coletti *et al*. 2001). It has been isolated in foals with pneumonia and suppurative infections, in mares with cervicitis and metritis, in cows with mastitis, and in dogs with wound infections, urinary tract infections, septicaemia and pneumonia (Carter and Wise 2005). *Klebsiella* infection in humans

can result in pulmonary and urinary tract disease (Coletti *et al*. 2001). Asymptomatic carriers may also have a role in an outbreak. *Klebsiella pneumoniae* has heightened prevalence in the immunosuppressed or in the presence of other infection (Coletti *et al*. 2001). Treatment should involve culture of the lesion and antimicrobial sensitivity testing of the isolate.

5.1.3.12 Lactobacillus sp.

Lactobacilli are Gram-positive, facultative, anaerobic bacteria (Biberstein and Zee 1990). They are part of the normal flora of the urogenital tract, and are commonly used to restore the bacterial balance of the gastrointestinal, urogenital and respiratory tracts. Lactobacilli can act competitively to exclude pathogens, inhibiting their colonisation and consequently preventing infection (Nader de Macias 1996). Many probiotic nutritional supplements have a high proportion of *Lactobacillus,* which is used to aid restoration of commensal intestinal microflora.

5.1.3.13 *Pasteurella* sp.

Pasteurella sp. are Gram-negative, non-motile, facultative coccobacilli (Biberstein and Zee 1990). Sporadic cases and small outbreaks of *Pasteurella multocida* have been reported in red kangaroos (Okoh 1980), a western-barred bandicoot (Phillips 1986) and wombats (Munday, unpublished). *Pasteurella* are carried on mucous membranes of the gastrointestinal and/or upper respiratory tracts of susceptible host species including ruminants, horses, swine, rabbits, fowl, dogs, cats, rodents and humans (Mannheim 1983). Diseases caused by *Pasteurella multocida* in production animals include bovine 'shipping fever' a fibrinous pleuro or bronchopneumonia typically associated with handling and transport: septicaemic pasteurellosis, enzootic pneumonia and gangrenous mastitis in sheep and goats: and fibrinous pneumonia and atrophic rhinitis in swine and piglets. 'Snuffles' a mucopurulent rhinosinusitis in rabbits, is typically associated with stressful conditions such as pregnancy and lactation, and can cause orchitis, balanoposthitis and pyometra in the genital tract. Fowl can develop fowl cholera, characterised either by sudden death or nasal and ocular discharges, anorexia, diarrhoea and respiratory signs. In dogs and cats *Pasteurella* are typically found in conjunction with other anaerobic flora in wound infections and foreign body lesions. While in horses, *Pasteurella* have been associated with *S.*

zooepidemicus in equine respiratory disease and in rodents opportunistic infections typically result in pneumonia (Biberstein and Zee 1990). Pasteurelloses are responsive to antibiotics pending culture and antimicrobial sensitivity results, and immunisation has only been found useful in bovine haemorrhagic septicaemia and in the control of atrophic rhinitis (Biberstein and Zee 1990).

5.1.3.14 Porphyromonas sp. and *Prevotella* sp.

Shah and Collins (1988) further defined some of the Gram-negative anaerobe *Bacteroides* based on saccharolytic qualities as *Porphyromonas* and *Prevotella* sp. Usually the black pigmented anaerobe *Porphyromonas* sp. inhabits the oropharynx, nose, gastrointestinal and urogenital tract in humans. However, it has also been isolated from a brain abscess, liver abscess (Jover-Diaz 2003), sinusitis, osteomyelitis (Duerden 1993), dental-oral bacteraemia, inflammatory pelvic disease, balanitis, endometritis and cutaneous and soft tissue infections (Melon *et al.* 1997). At least seven *Porphyromonas* sp. of animal origin, mostly from dogs, have also been described (Jover-Diaz 2003). *Prevotella* are common in the gastro-intestinal tract of humans and animals, many are found in the rumen and hindgut. Antimicrobial agents are chosen on the results of culture and antimicrobial sensitivity testing of the isolate.

5.1.3.15 Proteus sp.

Proteus are opportunistic Gram-negative, facultative pathogens, similar to other members of the family *Enterobacteriaceae* (Biberstein and Zee 1990). *Proteus mirabilis* has been implicated in a variety of infections in dogs, cats, cattle, fowl and other animals. Although considered an opportunistic invader, diarrhoea associated with *P. mirabilis* infection has been reported in young mink, hedgehogs, lambs, goats, calves and puppies (Carter and Wise 2004). In companion animals and humans, urinary tract infections with resultant struvite or arpatite stone formation in the kidneys have been seen. *P. mirabilis* infection is part of the commensal canine and hedgehog microflora, and these bacteria are generally found in small numbers unless the intestinal balance is disrupted (Isenbugel and Baumgartner 1993).

5.3.1.16 Pseudomonas sp.

Pseudomonas sp. Are aerobic Gram-negative rods. Three of the 27 recognised species of Pseudomonads have veterinary significance and these are *Pseudomonas aeruginosa, Burkholderia (Pseudomonas) mallei and Burkholderia (Pseudomonas) pseudomallei* (Hirsh 1990). *Pseudomonas* live in soil and water and are routinely found in animal faeces (Hirsh 1979). *Pseudomonas aeruginosa* tends to contaminate areas of the body with reduced numbers of normal flora, often post treatment with antimicrobials. Due to its general resistance against certain types of commonly used antibiotics, it will replace the normal flora and if epithelial compromise exists, colonisation and subsequent infection will occur (Biberstein and Zee 1990). However, it has also been isolated from animals with no history of antimicrobial therapy in cases of canine otitis externs and urinary tract infections. Treatment involves correction of the compromise. Culture and sensitivity is paramount to determine the appropriate use of antimicrobials as it is resistant to most antimicrobials except gentamicin.

5.1.3.16 *Serratia* sp.

Serratia are opportunistic Gram-negative bacteria classified in the tribe Klebsielleae and the family Enterobacteriaceae (Fernandez and Mochales 1994). *Serratia marcescens* is thought to be the most pathogenic species in humans and has been associated with nosocomial infections of the bloodstream, lower respiratory tract, urinary tract, surgical wounds, and skin and soft tissues of adult humans. Risk factors for severe infections with *Serratia* include old age, previous antibiotic treatment, and chronic or debilitating diseases (Ania 2007). Outbreaks of *S. marcescens* meningitis, wound infections, and arthritis have also occurred in pediatric wards (Mochales and Fernandez 1994). Culture of the organism and antimicrobial sensitivity is required as resistance is high for this organism (Ania 2007).

5.1.3.17 Spirochaetes – Borrelia, Brachyspira, Leptospira, and Treponema.

Spirochaetes belong to two familes, the family Spirochaetacea comprises *Borrelia, Brachyspira* and *Treponema,* and the family Leptospiraceae comprising *Leptospira. Borreliae* are 0.2 to 0.5μm by 8 to 30μm Gram-negative microaerophilic to anaerobic spirochaetes transmitted and

maintained mostly by ticks. *Borrelia* spirochaetes were found on a cloacal swab of a southern-brown bandicoot, eastern-grey kangaroo, and a red kangaroo in Queensland (Mackerras 1959). No clinical signs of infection were observed.

The intestinal anaerobic spirochaete *Brachspira pilosicoli* (formerly called *Serpulina*) was isolated from cloacal swabs in five bilbies (*Macrotis lagostis)* at Dryandra in Western Australia, and also from Dryandra bilbies on post mortem exam. The strain was typed by PCR to be the human species *Brachyspira* (*Serpulina) pilosicioli* (Warren 2002, unpublished report). No signs of ill health were noted in these individuals. *B. pilosicoli* causes colonic spirochaetosis in pigs and chickens as well as humans. The best known pathogenic agent of this species is *Brachyspira (Serpulina) hyodysenteriae*, the agent of swine dysentery (Biberstein and Zee 1990).

Treponemes are obligate anaerobic spirochaetes (Hirsh 1990) found in the oral cavity and genitals of humans, and in the intestinal tract and feet of animals (Edwards *et al.* 2003). In animals there is strong evidence that spirochaetes phylogenetically related to human oral *Treponema* species are involved in the pathogenesis of digital dermatitis (DD) in the bovine foot and contagious ovine digital dermatitis (CODD) in the ovine foot (Edwards *et al.* 2003). DD and CODD are infectious diseases often associated with lameness, excessive moisture, bleeding and eventual under running of the toe (Rijpkema *et al.* 1997). It is likely that DD and CODD originate as polymicrobial infections, and that conditions favouring growth of *Treponema* may be generated during the formation of the primary lesion. However, it is also possible that animal *Treponema* may exist commensally under non-disease conditions (Edwards *et al.* 2003).

Leptospires are thin, Gram-negative, motile spiral organisms 0.1µm by 6-20µm (Biberstein and Zee 1990). There are 13 geno species and over 250 serovars, many of which are of veterinary significance. Isolation and serological studies were undertaken on 600 bandicoots in Queensland (Emanuel and Mackerras 1964), and both the long-nosed bandicoot, and the golden bandicoot (*Isoodon macrurus)* were found to carry *Leptospira* sp., although no associated clinical disease was found. Munday and Corbould (1973) also found antibodies to *Leptospira interrogans* serovar

pomona in nine of 13 Tasmanian wombats. The affected individuals had an associated mild to severe interstitial nephritis, and were found in areas where the disease had been recently diagnosed in cattle. Experimental infection of two wombats caused depression, anorexia, icterus and death within 14 days, with interstitial nephritis and nephrosis being observed at necropsy (Munday and Corbould 1973). Antibody to *Leptospira pomona* was also reported by Munday (1972) in the eastern-barred bandicoot (*Permales gunnii)*, and to *Leptospira hyos* in the long-nosed potoroo.

5.1.3.18 *Salmonella* sp.

Salmonellosis is caused by the Gram-negative, facultative, bacterium *Salmonella* sp. and can lead to gastrointestinal signs of diarrhea, dysentery, depression, dehydration and possible septicaemia in macropods (Blyde 1999). *Salmonellae* are carried in the gastrointestinal tract and mesenteric lymph nodes of macropods (Speare 1988). Salmonellosis typically affects captive adult macropods and orphaned joeys (Speare 1988), and has not been reported in free ranging populations of macropods.

Salmonella has been isolated from the quokka along with many other Australian marsupials, including the eastern-grey wallaroo (*Macropus robustus*), tammar wallaby, red kangaroo, short-eared rock wallaby, western quoll, slender-tailed dunnart (*Sminthopsis murina)* and southern-brown bandicoot (Iveson and Bradshaw 1973). *Salmonella* sp. including *Salmonella chester*, *Salmonella eastbourne*, *Salmonella wandsbek* subsp II, *Salmonella birkenhead* and *Salmonella bahrenfeld* were cultured from cloacal swab in bilbies studied from 1999-2002 from Kanyana, Dryandra and the Pilbara in Western Australia. *Salmonella chester* was the most common strain, isolated from 37 individuals in the captive breeding colony in Northern Dryandra. No signs of ill-health were reported in these individuals. *Salmonella bahrenfield* was isolated from a wild bilby in poor condition in the Pilbara, however there was no evidence of diarrhea and the clinical significance of this infection remains unknown (Warren 2002, unpublished report).

An unusual abundance of *Salmonella* was detected in the West Australian Rottnest Island population of quokkas. In this locality, annual starvation in summer associated with significant mortality was reported (Hart, Bradshaw and Iveson 1985). Correspondingly high infection rates with *Salmonella* (70 to 100%) often associated with multiple serovars (up to four) were found over the summer period. At the end of summer, most animals excreted *Salmonella* sp. yet no evidence of clinical *Salmonella* related disease was seen. Only low infection rates (0 to 30%) were found over winter. The infections were believed to be associated with disruption of the digestive physiology of the animals caused by poor quality feed and were not directly linked to the severe end of summer decline in population (Hart, Bradshaw and Iveson 1985).

Low-level sub-clinical *Salmonella* infections are reported in domestic and wild animals (Taylor 1969). The promotion of infection by stress has also been extensively reviewed in humans (Rubin and Weinstein 1977), and also in mice suffering food and water deprivation (Tannock and Smith 1972), and cattle subjected to disease and environmental stress (Gibson 1965). In wild populations possible components or 'stressors' leading to disease could include lack of appropriate shelter and water, poor food quality, and/or inadequate quantity, and social issues. In the quokka, new infections resulted from reduced host defense and proliferation of existing infections (Hart, Bradshaw and Iveson 1985). These infections did not appear to exceed the threshold for clinical disease, however in the event of escalation of environmental stressors clinical disease may have become apparent (Hart, Bradshaw and Iveson 1985). Interestingly, diagnosis of salmonellosis in a child who had close contact with quokkas prompted much of this research (Iveson and Bradshaw 1973) demonstrating that quokkas can be a potential zoonotic reservoir.

Faecal culture provides definitive diagnosis, although due to intermittent shedding, multiple samples should be tested. In quokkas, Hart, Bradshaw and Iveson (1985) found faecal samples to be more effective than rectal swabs for detecting isolations in which small numbers of organisms were involved. Alternatively, immediate post-mortem culture of stomach contents,

faeces, heart, lung, spleen, lymph nodes, and gallbladder provides definitive diagnosis. The latter technique has obvious constraints when dealing with endangered populations.

Treatment typically involves antimicrobials, fluid and electrolyte therapy. Antibiotic therapy should be based on culture and antimicrobial sensitivity findings. Commonly used antibacterials include Ceftiofur 2mg/kg IM s.i.d 7-10 days or Enrofloxacin 5mg/kg IM SID for 7-10 days. Concurrent treatment with an antifungal is usually also undertaken to prevent *Candida* sp. overgrowth (Blyde 1999).

5.1.3.20 Staphylococcus sp.

Staphylococci are spherical Gram-positive cocci, which are often found in exudates and form clusters, pairs or short chains (Biberstein and Zee 1990). They are assumed to be present in the upper respiratory tract and on the epithelial surfaces of all warm-blooded animals (Blobel and Bruckler 1984). *S. aureus* is a common pyogenic agent in humans and several animal species. *S. intermedius* is the prominent pyogenic agent in canine species and *S. epidermidis* is present on skin and mucous membranes, though is rarely pathogenic. *S. hyicus* has been reported to cause exudative epidemitis of swine and has been involved in bovine mastitis (Biberstein 1990).

In marsupials, non-haemolytic staphylococci were cultured from a cloacal swab in one of 420 bilbies studied from 1999-2002 from Kanyana, Dryandra and the Pilbara in Western Australia. No signs of ill health were noted (Warren 2002, unpublished report).

5.1.3.21 Yersinia sp.

Yersinia are Gram-negative coccobacilli belonging to the family *Enterobacteriacea* (Biberstein and Zee 1990). *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* were reported by Munday (1988) to cause severe enteritis, leading to dehydration and sudden death in possums. Chronically infected possums displayed wasting. Histologically, multifocal, pale, necrotic foci were present in the liver, spleen and mesenteric lymph nodes with associated enteritis. In farmed

possums, predisposing causes included cold weather, change of feeding regime and diet, and capture stress.

5.1.4 Specific bacteria causing urogenital disease in Gilbert's potoroos

Retrospective analysis of medical records indicates there has been a long history of balanoposthitis (inflammation of the penis and prepuce) in both the wild and captive population of the Gilbert's potoroo. Clinically this is evident as crusty green preputial exudates. Bacteriological examination revealed a number of potential pathogens amongst the mixed bacteria isolated. However, as this condition was seen in both the wild and captive population, no treatment was initially thought to be required other than cleaning the severely affected animals with an antiseptic solution (Forshaw and Horwitz 2001, unpublished data).

One of the original founder potoroos, GP M3 was diagnosed with balanoposthitis in late August 2001. The veterinary clinical records of Dr Anne-Marie Horwitz stated 'thick black sand was evident adhered to the penis and underneath a very bright green discharge was seen. The penis was reddened and slightly swollen.' Other significant findings included weight loss which had been evident over the past 2 months and decreased appetite. A preputial swab taken at the time indicated a high number of spirochaete organisms. In early September 2001 despite two intramuscular injections of 0.1mL long acting penicillin, (prescribed to control the spirochaete organism), weight loss continued (30g weight loss over two months). Appetite was seen to improve, and the penis discharge was still evident although it was no longer bright green. The prepuce was again swabbed and on this occasion spirochaetes were not seen. A further long-acting penicillin injection was given.

By mid-September 2001 the potoroos weight had increased 25g, however the preputial discharge was still present. In mid-October the preputial discharge had again become fluorescent green in colouration. Two preputial swabs taken revealed large numbers of spirochaete organisms, which were unable to be cultured by conventional techniques. The veterinary pathologist, Dr David

Forshaw, commented that the spirochaetes were *Brachyspira*-like in morphology. A moderate growth of non-identifiable Gram-negative rods and *Actinobacillus* sp. were also seen. These other organisms have been seen before in both normal and abnormal animals and so no treatment was instigated. This animal was transferred to the Perth Zoo in mid-November. Three preputial swabs were taken, and spirochaetes resembling *Treponema* were present. Dr Nicky Buller, a microbiologist at DAFWA began DNA extraction, amplification and sequencing of the spirochaete. Culture was again attempted however, was unsuccessful as spirochaetes tend to be overgrown by other flora within a few days and are notoriously difficult to subculture. Dr Buller noted that the spirochaetes seen weren't motile. This was thought to be unusual as most *Treponema* have motility of some sort, and therefore it was assumed that the culture medium did not support the culture of these organisms.

Final sequencing results indicated a 164 nucleotide segment of ribosomal DNA. It had a 92% sequence homology to *Treponema* species, but no identification to any known species was made.

A female Gilbert's Potoroo transferred to the Perth Zoo (A01671) was also found to have a spirochaete on cloacal swab in mid June 2002. No other significant isolates were found on routine culture. Prior cloacal swabs in early June had revealed significant anaerobic growth, and moderate growth of *Streptococcus* sp. and *Bacteroides* sp. No evidence of spirochaetes of *Brachyspira* sp. had been seen previously.

The analogous long-nosed potoroos residing at the Perth Zoo had never been observed to have a green preputial discharge, and an extensive review of the literature substantiated these findings.

5.2 MATERIALS AND METHODS

Initial health assessment was carried out by visually checking for faecal soiling, urogenital discharge, assessment of body weight and body condition scoring. Complete health assessment was carried out under general anaesthesia. A physical and clinical examination, including cloacal swabs and blood collection for a general health profile was undertaken.

The Gilbert's potoroos were first anaesthetised. In males, the penis was exteriorized from its preputial sheath and observed for exudates, swelling and erythema. Similar observations were then made for the preputial sheath. The level and nature of discharge was then assigned a grade based on its severity (Appendix 4).

0 no discharge or swelling evident
1+ mild preputial swelling and exudate
2+ moderate preputial swelling and exudate
3+ severe preputial swelling and exudate.

In females, it was more difficult to classify the nature of discharge owing to their urogenital anatomy. As such, females were assigned as having no discharge or having the presence of discharge, the nature of which was commented on.

In both males and females, the urogenital opening within the cloaca was swabbed. In females the rectum lies dorsal to the urogenital opening, while in males the rectum lies dorsal to the penis (Tyndale-Biscoe and Renfree 1987). A dry swab (Copan-USA) was taken from the prepuce of males for *Treponema* molecular studies (DNA amplification via PCR). Following this a sterile Amie's swab, moistened with transport medium (Transwab®, Medical Wire and Equipment, England) was rolled under the prepuce for microbiological culture and speciation. A wet preparation was also made from this swab for dark field microscopy and the identification of spirochaetes. In the females, a dry sterile swab (Copan-USA) was taken from the urogenital

sinus for *Treponema* molecular studies (DNA amplification via PCR). Following this a second dry swab (Copan-USA) moistened, then placed in Amie's transport medium was used. The Transwab® (Medical Wire and Equipment, England) swab was not used due to the small diameter of the female orifice. Again a wet preparation was also made from this second swab for dark field microscopy and the identification of spirochaetes. These swabs were then forwarded onto DAFWA for microbiological analysis, culture and speciation via overnight courier on ice. *Treponema* was identified using dark field microscopy. Morphologically these organisms were typically smaller than *Brachyspira* with tighter spirals. In total, the *Treponema* organism had eight tight spirals.

A moistened Amie's transport medium swab (Transwab®, Medical Wire and Equipment, England) was also collected in both males and females from the rectal opening of the cloaca for microbial culture, including *Salmonella* testing, and couriered overnight on ice to Murdoch University for culture and *Salmonella* typing (if present).

The bladder was palpated and manually expressed. A mid-stream, free-catch sample was collected and placed into a sterile urine container and then forwarded via overnight courier on ice to Murdoch University for urinalysis, and sediment exam. Culture was undertaken if large numbers of inflammatory cells were evident microscopically.

Results were tabulated to determine the most prevalent microbes found in association with the *Treponema* organism. The statistics program SPSS® (Chicago, Illinois) was then used to check for statistical significance between certain microbes and the presence of *Treponema*. Distribution of continuous data was inspected for normality through the generation of histograms and calculation of skewness and kurtosis values, and Levene's test was performed to examine for homogeneity of variance between groups (when applicable). Odds ratios (OR) were used for the analysis of data when significant differences between groups were observed. In many cases Yates corrections for continuity were employed to compensate for the over-estimate of the chi-square when used with a two by two table.

Over the course of the study 34 individuals had a swab taken of the rectal opening of the cloaca and 44 individuals had a swab taken from the urogenital opening of the cloaca. Given the estimated population of the Gilbert's potoroo is only 35 individuals, these sample sizes are more reflective of a census rather than a population estimate as seen in the majority of studies concerning statistics.

It should also be remembered that in small population sizes, results that have clinical (biological) significance may not have statistical significance. The effect of a small sample size can make it difficult to draw sound conclusions with regards to such issues and potentially result in genuine effects going unnoticed. Therefore, the results of the health screening of Gilbert's potoroos, should be interpreted with due reference to potential clinical significance in this critically endangered species.

5.3 RESULTS

A chart grading the severity of associated lesions seen was formulated for ease of description for ongoing monitoring of the level of discharge and associated cloacal and preputial inflammation (Appendix 4).

5.3.1 Cloacal swab of the rectal orifice

In total, 73 swabs were cultured from 34 individuals over the course of the study. This comprised seven captive potoroos (four females and three males) and 27 wild individuals (14 females and 13 males). Thirteen species of bacteria were isolated from the rectal orifice within the cloaca in male and female Gilbert's potoroos (Appendix 5). Many swabs revealed mixed infections. No evidence of *Salmonella* infection was found.

5.3.1.1 Comparisons: seasonality and species identified (Appendix 5)

- The most commonly isolated species included coryneform bacilli*, E. coli* and an α-*alpha haemolytic Streptococcus* sp. which were found in individuals over all trapping sessions (100%).

- Of these three species, *E.coli* was isolated in an equal or higher prevalence at every trapping session.

- *Enterococcus* sp. was present in individuals in four of the five trapping sessions (80%). *Bacillus* sp**.** *Proteus mirabilis, Pseudomonas sp.* and non-haemolytic *Streptococcus* sp.*,* were present in individuals in three of the five trapping sessions (60%).

- *Pasteurella* sp. *Serratia liquefaciens,* coagulase positive *Staphylococcus* sp. and coagulase-negative *Staphylococcus* sp. were present in individuals in two of the five trapping sessions (40%). ß-haemolytic *Streptococcus* sp. was only present in one individual in one of the five trapping sessions (20%).

- The highest variation in number of bacterial species identified was found in the June 2006 trapping session (winter) with 10 of the identified 13 species (76.9%) being cultured. The lowest variation in the number of species identified was the June 2005 trapping session (winter).

- The greatest number of bacterial isolates from multiple species was recorded in the November 2005 trapping session (27) the lowest number of bacterial isolates from multiple species was found in June 2005 (11).

5.3.1.2 Comparisons: seasonality, and sex (Table 5.1)

- A greater or equal variation in bacterial species was found in males, when compared to females in all trapping sessions other than November 2005.

- A greater number of isolates from multiple species was found in males at all trapping sessions.

- *Pasteurella* sp. *Serratia liquefaciens, coagulase positive Staphylococcus* spp*.* and *β beta-haemolytic Streptococcus spp.* were only isolated from males and were of low prevalence.

- *E. coli* was present in a greater number of female individuals than males in all trapping sessions other than November 2006.

Table 5.1 Seasonal rectal microbiology findings in male compared to female individuals.

Microbes found in Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 15)		November 2005 (n = 18)		June 2006 (n = 15)		November 2006 (n = 12)	
Sex affected	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
N =	**8**	**5**	**9**	**6**	**10**	**8**	**10**	**5**	**7**	**5**
***Bacillus* sp.**	1/12 8.3%	1/7 14.3%					2/20 10%		1/9 11.1%	1/8 12.5%
Coryneform bacilli	2/12 16.7%		1/6 16.7%	1/5 20%	1/15 6.7%	2/12 16.7%	4/20 20%	2/6 33.3%	2/9 22.2%	
***Enterococcus* sp.**		2/7 28.6%	2/6 33.3%	1/5 20%	2/15 13.3%	2/12 16.7%				3/8 37.5%
E.coli	1/12 8.3%	2/7 28.6%	1/6 16.7%	2/5 40%	4/15 26.7%	2/12 16.7%	4/20 20%	2/6 33.3%	1/9 11.1%	4/8 50%
Pasteurella	2/12 16.7%						1/20 5%			
Proteus mirabilis	1/12 8.3%	1/7 14.3%				2/12 16.7%	1/20 5%	1/6 16.7%		
***Pseudomonas* sp.**	2/12 16.7%	1/7 14.3%				1/12 8.3%	1/20 5%			
Serratia liquefaciens					1/15 6.7%				1/9 11.1%	
Staphylococcus* sp. *Coagulase +	1/12 8.3%						1/20 5%			
Staphylococcus* sp. *Coagulase -						1/12 8.3%			1/9 11.1%	
Streptococcus* sp. *α-haemolytic	2/12 16.7%		2/6 33.3%	1/5 20%	4/15 26.7%	1/12 8.3%	3/20 15%	1/6 16.7%	1/9 11.1%	
Streptococcus* sp. *non-haemolytic					3/15 20%	1/12 8.3%	2/20 10%		2/9 22.2%	
Streptococcus* sp. *β-haemolytic							1/20 5%			
Number of species in total (diversity)	**8**	**5**	**4**	**4**	**6**	**8**	**10**	**4**	**7**	**3**
Number of isolates in total	**12**	**7**	**6**	**5**	**15**	**12**	**20**	**6**	**9**	**8**

5.3.1.3 Comparisons: seasonality and species identified in captive and wild individuals (Table 5.2)

- A greater diversity of species was isolated from the rectal opening within the cloacal orifice over all trapping sessions in the wild compared to the captive population. Coryneform bacilli, *Serratia liquefaciens* and coagulase-positive *Staphylococcus* sp. were only found in the wild population and *Proteus mirabilis, Pseudomonas* sp. were only found in the captive population.

- In the captive colony *E. coli* was the most frequently isolated microbe found in the rectal orifice of the cloaca (50% of captive individuals had evidence of *E. coli* in June and November 2006).

- *Pseudomonas* sp. was isolated from 40% of captive individuals in March 2005.

- In the wild population, *E. coli* was the most frequently isolated microbe found in the rectal orifice of the cloaca (38.5% of the wild population had evidence of *E. coli* in June 2005).

Table 5.2 Seasonal rectal microbiology findings in the captive compared to the wild population.

Microbes found in Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 15)		November 2005 (n = 18)		June 2006 (n = 15)		November 2006 (n = 12)	
Population captive (C) or wild (W) n =	**C 4**	**W 9**	**C 6**	**W 9**	**C 6**	**W 12**	**C 4**	**W 11**	**C 4**	**W 8**
***Bacillus* sp.**	1/10 10%	1/9 11.1%						1/17 5.9%		2/17 11.7%
Coryneform bacilli		2/9 22.2%		2/13 15.4%		2/18 11.1%		4/17 23.5%		4/17 23.5%
***Enterococcus* sp.**	1/10 10%	1/9 11.1%		3/13 23.1%		2/18 11.1%		1/17 5.9%	1/6 16.7%	2/17 11.7%
E. coli	2/10 20%	1/9 11.1%		5/13 38.5%	3/9 33.3%	5/18 27.8%	4/8 50%	5/17 29.4%	3/6 50%	4/17 23.5%
Pasteurella	1/10 10%	2/9 22.2%								
Proteus mirabilis	1/10 10%				2/9 22.2%		2/8 25%			
***Pseudomonas* sp.**	4/10 40%				1/9 11.1%					
Serratia liquefaciens										2/17 11.7%
Staphylococcus* sp. *Coagulase +		1/9 11.1%				1/18 5.5%		2/17 11.7%		
Staphylococcus* sp. *Coagulase -						1/18 5.5%			1/6 16.7%	
Streptococcus* sp. *α-haemolytic		1/9 11.1%		3/13 23.1%	3/9 33.3%	3/18 16.7%	1/8 12.5%	2/17 11.7%		2/17 11.7%
Streptococcus* sp. *non-haemolytic						4/18 22.2%	1/8 12.5%	1/17 5.9%	1/6 16.7%	1/17 5.9%
Streptococcus* sp. *β-haemolytic								1/17 5.9%		
Number of species in total (diversity)	**6**	**7**	**0**	**4**	**4**	**7**	**4**	**8**	**4**	**7**
Number of isolates in total	**10**	**9**	**0**	**13**	**9**	**18**	**8**	**17**	**6**	**17**

5.3.2 Cloacal swab of the urogenital orifice

In total, 108 swabs were cultured from 39 individuals over the course of the study. This number was greater than the number collected from the rectal orifice, as a further trapping session in March 2007 was undertaken specifically to collect urogenital swabs to increase sample size and further investigate the significance of the *Treponema* infection in relation to resident microflora. The 108 swabs collected were from eight captive potoroos, (five males and three females) and 31

wild individuals (14 males and 17 females) (Appendix 6). 100 swabs were also collected for PCR for *Treponema* infection. The disparity of eight between swabs for culture and swabs for PCR was owing to the courier service losing samples.

In total, 23 aerobic bacteria and 11 anaerobic bacteria were isolated from the urogenital orifice of the cloaca. Many swabs revealed mixed infections. No evidence of *Salmonella* infection was found.

Given that multiple methods to diagnose *Treponema* were undertaken the following criteria were used to determine infection status. Male and female potoroos were classified as infected if they were PCR positive, and had evidence of spirochaetes on dark field microscopy, in conjunction with urogenital discharge. Additionally the presence of characteristic spirochaetes seen on dark field microscopy even in the absence of PCR positive results, where discharge was present, was assumed to be indicative of infection. Females, which were PCR negative and lacked discharge yet had spirochaetes seen on dark field microscopy, were classified as infected.

5.3.2.1 Comparisons: seasonality and aerobic species identified (Appendix 7)

- The most commonly isolated species included *Actinobacillus* sp*. Corynebacterium pilosum* and *Pasteurella* sp. which were found in individuals over all trapping sessions (100%).

- Of these three species *Pasteurella* sp. was isolated in an equal or higher prevalence at every trapping session other than in November 2006 and March 2007 where *Actinobacillus* had the highest prevalence.

- *Brackiella oedipus* was found in five of the six trapping sessions (83.3%) while *Bacillus* sp., *Pseudomonas aeruginosa,* non-haemolytic *E. coli* and *Staphylococcus* sp. were present in individuals in four of the six trapping sessions (66.7%).

- *Enterobacter faecalis*, a non-fermentative Gram-negative rod, and *Staphylococcus aureus* were isolated in three of the six trapping sessions (50%).

- The coliforms, *Enterobacter cloacae, Klebsiella oxytoca, Pantoea* sp.*,* and *Serratia marcescens* were isolated in two of the six trapping sessions (33.3%).

- *Enterobacter aerogenes, Enterobacter vulneris*, haemolytic *E. coli, Lactobacillus, Proteus vulgaris, Serratia liquefaciens and Staphylococcus epidermidis* were isolated from only one of the six trapping sessions (16.7%).

- The highest variation in the number of species identified was found in June 2006 trapping session (winter) where 16 species were isolated, interestingly this is the same trapping session where the highest diversity in bacteria was found in the rectal opening of the cloaca.

- The lowest variation in the number of species identified was the March 2005 trapping session (autumn) where only eight species were isolated.

- The greatest number of bacterial isolates (n=53) was recorded in the March 2007 trapping session, the lowest number of bacterial isolates (n=11) was found in March 2005.

5.3.2.2 Comparisons: seasonality and anaerobic species identified (Appendix 8)

- The most commonly isolated anaerobic species was *Treponema,* identified through PCR, and dark field microscopy in all trapping sessions (100%). All other anaerobes were identified through culture methods.

- *Prevotella* was the second most commonly isolated anaerobic species identified in five trapping sessions (83.3%).

- *Clostridium glycolicum* and *Porphyromonas asaccharolytica* were isolated in four of the six trapping sessions (66.7%).

- *Bacteroides fragilis, Bacteroides melaninogenicus, Bacteroides thetaiotaomicron, Bifidobacterium* sp. *Eubacterium lentum, Fusobacterium* and *Helcoccus* were isolated from only one of the six trapping sessions (16.7%).

- The highest variation in the number of species identified was in the June 2006 and November 2006 sessions where five species were isolated. Four species were isolated from all other trapping sessions.

- The greatest number of bacterial isolates was recorded in the June 2006 trapping session (n=24) the lowest number of bacterial isolates was found in March 2005 (n=11).

5.3.2.3 Comparisons: seasonality and sex in aerobic bacteria findings in the urogenital orifice of the cloaca in the Gilbert's potoroo (Table 5.3, Appendix 9)

- Of the three most prevalent aerobes, *Actinobacillus* sp*, Corynebacterium pilosum and Pasteurella* sp*., Actinobacillus* sp. was found in males at every trapping session compared to three out of six sessions (50%) for females and had a higher prevalence in all trapping sessions in males.

- There was no discernible difference between prevalence of *Corynebacterium pilosum* in males compared to females.

- *Pasteurella* sp. was found in males and females at every trapping session and had a higher prevalence in all trapping sessions in males.

- *Enterobacter cloacae, E. coli* (haemolytic), *Lactobacillus, Proteus vulgaris* and *Staphylococcus epidermidis* were only present in females.

- *Enterobacter aerogenes, Enterobacter vulneris, Pantoea* sp*., Serratia marscescens and Serratia liquefaciens* were only present in males.

- A greater number of isolates were present in males as compared to females across all trapping sessions other than November 2006.

- A greater number of microbial species was present in males in March 2005, June 2005 and June 2006, while in November 2005, November 2006 and March 2007 a greater number of species was isolated from females.

Table 5.3 Seasonal urogenital aerobic microbiology findings in male compared to female individuals.

Aerobic microbes found in Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 14)		November 2005 (n = 20)		June 2006 (n = 11)		November 2006 (n = 13)		March 2007 (n=22)		Number of sessions bacteria found	
Sex affected	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
n =	**6**	**7**	**9**	**5**	**10**	**10**	**7**	**4**	**7**	**6**	**10**	**12**		
***Actinobacillus* sp.**	1/8 12.5%		1/22 4.5%		9/26 34.6%	2/16 12.5%	4/24 16.6%		5/17 11.8%	2/23 8.7%	10/27 37%	3/26 11.5%	**6/6 100%**	**3/6 50%**
***Bacillus* sp.**			1/22 4.5%		1/26 3.8%				1/17 11.8%	1/23 4.3%		1/26 3.8%	**3/6 50%**	**2/6 33.3%**
Brackiella oedipus		1/4 25%			2/26 7.7%	5/16 31.2%	2/24 8.3%	1/11 9.1%	1/17 11.8%	6/23 26.1%	1/27 3.7%	7/26 26.9%	**4/6 66.6%**	**5/6 83.3%**
Coliforms	1/8 12.5%					1/16 6.2%							**1/6 16.7%**	**1/6 16.7%**
Corynebacterium pilosum.	1/8 12.5%	1/4 25%	4/22 18.2%	1/8 12.5%	5/26 19.2%	3/16 18.7%	3/24 12.5%	2/11 18.2%	2/17 11.8%	2/23 8.7%	1/27 3.7%	3/26 11.5%	**6/6 100%**	**6/6 100%**
Enterobacter aerogenes	1/8 12.5%												**1/6 16.7%**	
Enterobacter Cloacae				1/8 12.5%				1/11 9.1%						**2/6 33.3%**
Enterobacter Faecalis								2/11 18.2%		3/23 13%	2/27 7.4%	3/26 11.5%	**1/6 16.7%**	**3/6 50%**
Enterobacter Vulneris	1/8 12.5%												**1/6 16.7%**	
E.coli (haemolytic)										1/23 4.3%				**1/6 16.7%**
E.coli (non-haemolytic)			2/22 9.1%	1/8 12.5%			2/24 8.3%			2/23 8.7%	3/27 11.1%	5/26 19.2%	**3/6 50%**	**3/6 50%**
Klebsiella oxytoca				1/8 12.5%					2/17 11.8%	1/23 4.3%			**1/6 16.7%**	**2/6 33.3%**
Lactobacillus								1/11 9.1%						**1/6 16.7%**
Non-fermentive GNR			4/22 18.2%	1/8 12.5%			1/24 4.2%	1/11 9.1%	1/17 5.8%	1/23 4.3%			**3/6 50%**	**3/6 50%**
Pantoea sp.			1/22 4.5%								1/27 3.7%		**2/6 33.3%**	
Pasteurella sp.	3/8 37.5%	1/4 25%	5/22 22.7%	1/8 12.5%	9/26 34.6%	3/16 18.7%	8/24 33.3%	1/11 9.1%	4/17 23.5%	1/23 4.3%	9/27 33.3%	3/26 11.5%	**6/6 100%**	**6/6 100%**
Proteus vulgaris				1/8 12.5%										**1/6 16.7%**
Pseudomonas aeruginosa			1/22 4.5%			1/16 6.2%	1/24 4.2%					1/26 3.8%	**2/6 33.3%**	**2/6 33.3%**
Serratia marcescens							1/24 4.2%		1/17 5.8%				**2/6 33.3%**	
Serratia liquefaciens							1/24 4.2%						**1/6 16.7%**	
***Staphylococcus* sp.**		1/4 25%		1/8 12.5%			1/24 4.2%		1/17 5.8%	2/23 8.7%			**2/6 33.3%**	**3/6 50%**
Staphylococcus aureus			3/22 13.6%			1/16 6.2%				1/23 4.3%			**1/6 16.7%**	**2/6 33.3%**
Staphylococcus epidermidis								1/119. 1%						**1/6 16.7%**
Number of species in total	**6**	**4**	**9**	**8**	**5**	**7**	**10**	**8**	**9**	**12**	**7**	**8**		
Number of isolates in total	**8**	**4**	**22**	**8**	**26**	**16**	**24**	**11**	**17**	**23**	**27**	**26**		

5.3.2.4 Comparisons: seasonality, sex and anaerobic bacteria (Table 5.4, Appendix 9)

- The most prevalent anaerobic species found in every trapping session *(Treponema)* was found in both males and females at every trapping session.

- *Bacteroides fragilis*, *Bacteroides melaninogenicus, Eubacterium lentum, Fusobacterium*, and *Helcococcus* were only found in males. *Bifidobacterium* sp. was only found in one female. All other listed anaerobes were found in both males and females.

- The total number of bacterial species and isolates from males was greater or equal to females at all trapping sessions.

Table 5.4 Seasonal urogenital anaerobic microbiology findings in male compared to female individuals.

Anaerobic bacteria found in Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 14)		November 2005 (n = 20)		June 2006 (n = 11)		November 2006 (n = 13)		March 2007 (n=22)		Number of sessions bacteria found	
Sex affected	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
n =	**6**	**7**	**9**	**5**	**10**	**10**	**7**	**4**	**7**	**6**	**10**	**12**		
Bacteroides fragilis									1/12 8.3%				**1/6 16.7%**	
Bacteroides melaninogenicus			1/12 8.3%										**1/6 16.7%**	
Bacteroides thetaiotaomicron	1/7 14.3%	1/4 25%											**1/6 16.7%**	**1/6 16.7%**
***Bifidobacterium* sp.**												1/5 20%		**1/6 16.7%**
Clostridium glycolicum			1/12 8.3%		1/12 8.3%		2/19 10.5%	1/5 20%	1/12 8.3%	1/8 12.5%			**4/6 66.7%**	**2/6 33.3%**
Eubacterium lentum							2/19 10.5%						**1/6 16.7%**	
***Fusobacterium* sp.**											1/13 7.7%		**1/6 16.7%**	
Helcococcus	1/7 14.3%												**1/6 16.7%**	
Porphyromonas asaccharolytica			1/12 8.3%		1/12 8.3%	3/10 30%	1/19 5.3%	1/5 20%	3/12 25%	3/8 37.5%			**4/6 66.7%**	**3/6 50%**
Prevotella		1/4 25%			1/12 8.3%		5/19 26.3%	1/5 20%	1/12 8.3%	3/8 37.5%	3/13 23.1%		**4/6 66.7%**	**3/6 50%**
***Treponema* – infected**	5/7 71.4%	2/4 50%	9/12 75%	1/1 100%	9/12 75%	7/10 70%	9/19 47.4%	2/5 40%	6/12 50%	1/8 12.5%	9/13 69.2%	4/5 80%	**6/6 100%**	**6/6 100%**
Number of species in total	**3**	**3**	**4**	**1**	**4**	**2**	**5**	**4**	**5**	**4**	**3**	**2**		
Number of isolates in total	**7**	**4**	**12**	**1**	**12**	**10**	**19**	**5**	**12**	**8**	**13**	**5**		

5.3.2.5 Comparisons: Seasonal aerobic microbiology findings in the urogenital orifice of the cloaca in the Treponema infected compared to the non-Treponema infected Gilbert's potoroo population (Table 5.5, Appendix 10)

- *Actinobacillus, Corynebacterium pilosum* and *Pasteurella* sp. were isolated from every trapping session in the *Treponema* infected population.

- Of these three species *Pasteurella* sp. was isolated in an equal or higher prevalence at every trapping session other than November 2006 and March 2007 where *Actinobacillus* had the highest prevalence.

- In the non-*Treponema* infected population *Actinobacillus* was only isolated in four of six (66.7%) trapping sessions, likewise *Corynebacterium pilosum* and *Pasteurella* were only isolated from five of six (83.3%) trapping sessions.

- *Brackiella oedipus,* was found in four of the six trapping sessions (66.7%) in the *Treponema* infected population. *Bacillus,* non-haemolytic *E. coli,* and a non-fermentative Gram-negative rod were present in individuals in three of the six trapping sessions (50%)

- *Klebsiella oxytoca*, *Pantoea*, *Pseudomonas aeruginosa, Serratia marcescens Staphylococcus aureus* and *Staphylococcus* sp. were isolated in two of the six trapping sessions (33.3%) and the coliforms*, Enterobacter aerogenes, Enterobacter cloacae, Enterobacter faecalis, Enterobacter vulneris*, *and Serratia liquefaciens* were isolated from only one of the six trapping sessions (16.7%) in the *Treponema* infected population.

- Haemolytic *E. coli, Lactobacillus, Proteus vulgaris* and *Staphylococcus epidermidis* were only found in the non-*Treponema* infected population.

- The highest variation in the number of species identified in the *Treponema* infected population was 11 species found in the June 2005 trapping session (winter), whereas the highest variation in species in the non-*Treponema* population was 11 species found in the November 2006 (spring) trapping session.

- The lowest variation in the number of species identified was the November 2005 trapping sessions (spring) where only six species were isolated compared to the non-*Treponema* infected population where only three species were isolated in March 2005.

- In the *Treponema* infected population, the greatest number of bacterial isolates was recorded in the November 2005 trapping session (n=32) whereas the lowest number of bacterial isolates was recorded in March 2005 (n=7). In the non-*Treponema* infected population the greatest number of bacterial isolates was recorded in the March 2007 trapping session (n=27), whereas the lowest number of bacterial isolates was recorded in March 2005 (n=5).

- SPSS® (Chicago, Illinois) univariate analysis was undertaken. The number of individuals with *Actinobacillus* and *Treponema* infection was found to be significantly different from the numbers of individuals with *Actinobacillus* that were not infected with *Treponema* (n = 100, Chi-Square test, p = 0.00, OR 5.824 95% C.I. 2.009-16.882). The *Treponema* infected population was 5.824 times more likely to have *Actinobacillus* infection than the non-*Treponema* infected population.

- The number of individuals with *Pasteurella* and *Treponema* infection was found to be significantly different from the numbers of individuals with *Pasteurella* that were not infected with *Treponema* (n = 100, Chi-Square test, p = 0.011, OR 3.343 95%C.I.1.385-8.066). The *Treponema* infected population was 3.343 times more likely to have *Pasteurella* than the non-*Treponema* infected population.

- The number of individuals with *Coynebacterium pilosum* and *Treponema* infection was not significantly different from the numbers of individuals with *Coynebacterium pilosum* that were not infected with *Treponema* (n = 100, Chi-Square test, p = 1).

Table 5.5 Seasonal urogenital aerobic microbiology findings in the non-*Treponema* infected Gilbert's potoroo population.

Aerobic microbes found in Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 14)		November 2005 (n = 20)		June 2006 (n = 11)		November 2006 (n = 13)		March 2007 (n=22)		Number of sessions bacteria found	
***Treponema* Infected (I) or non-infected (NI)**	**I**	**NI**	**I**	**NI**	**I**	**NI**	**I**	**NI**	**I**	**NI**	**I**	**NI**	**I**	**NI**
***Actinobacillus* sp.**	1/7 14.3%		1/25 4%		9/32 28.1%	2/9 22.2%	3/1717 .6%	1/18 5.6%	6/20 30%	1/20 5%	9/26 34.6%	4/27 14.8%	**6/6 100%**	**4/6 66.7%**
***Bacillus* sp.**			1/25 4%		1/32 3.1%				1/20 5%	1/20 5%		1/27 3.7%	**3/6 50%**	**2/6 33.3%**
Brackiella oedipus	1/7 14.3%				4/32 12.5%	3/9 33.3%		3/18 16.7%	2/20 10%	5/20 25%	3/26 11.5%	5/27 18.5%	**4/6 66.7%**	**4/6 66.7%**
Coliforms	1/7 14.3%							1/185. 6%					**1/6 16.7%**	**1/6 16.7%**
Corynebacterium pilosum.	1/7 14.3%	1/5 20%	4/25 16%	1/5 20%	7/32 21.8%	1/9 11.1%	1/175. 9%	4/1822 .2%	2/20 10%	2/20 10%	2/267. 7%	2/27 7.4%	**6/6 100%**	**5/6 83.3%**
Enterobacter aerogenes	1/7 14.3%												**1/6 16.7%**	
Enterobacter cloacae			1/25 4%					1/185. 6%					**1/6 16.7%**	**1/6 16.7%**
Enterobacter Faecalis								2/1811 .1%		3/20 15%	2/267. 7%	3/27 11.1%	**1/6 16.7%**	**3/6 50%**
Enterobacter Vulneris	1/7 14.3%												**1/6 16.7%**	
E. coli (haemolytic)										1/20 5%				**1/6 16.7%**
E. coli (non-haemolytic)			2/25 8%	1/5 20%			1/175. 9%	1/185. 6%		2/20 5%	1/263. 8%	7/27 25.9%	**3/6 50%**	**4/6 66.7%**
Klebsiella oxytoca			1/25 4%						1/20 5%	2/20 10%			**2/6 33.3%**	**1/6 16.7%**
Lactobacillus								1/185. 6%						**1/6 16.7%**
Non-fermentive GNR			4/25 16%	1/5 20%			2/1711 .7%		2/20 10%				**3/6 50%**	**1/6 16.7%**
***Pantoea* sp.**			1/25 4%								1/263. 8%		**2/6 33.3%**	
***Pasteurella* sp.**	1/7 14.3%	3/5 60%	6/25 24%		10/32 31.2%	2/9 22.2%	6/1735 .3%%	3/1816 .7%	4/20 20%	1/20 5%	8/2630 .7%	4/27 14.8%	**6/6 100%**	**5/6 83.3%**
Proteus vulgaris				1/5 20%										**1/6 16.7%**
Pseudomonas aeruginosa			1/25 4%			1/9 11.1%	1/175. 9%					1/27 3.7%	**2/6 33.3%**	**2/6 33.3%**
Serratia marcescens							1/17 5.9%		1/20 5%				**2/6 33.3%**	
Serratia liquefaciens							1/175. 9%						**1/6 16.7%**	
***Staphylococcus* sp.**		1/5 20%		1/5 20%			1/175. 9%		1/20 5%	1/20 5%			**2/6 33.3%**	**3/6 50%**
Staphylococcus aureus			3/25 12%		1/32 3.1%					1/20 5%			**2/6 33.3%**	**1/6 16.7%**
Staphylococcus epidermidis								1/185. 6%						**1/6 16.7%**
Number of species in total	**7**	**3**	**11**	**5**	**6**	**5**	**9**	**10**	**9**	**11**	**7**	**8**		
Number of isolates in total	**7**	**5**	**25**	**5**	**32**	**9**	**17**	**18**	**20**	**20**	**26**	**27**		

5.3.2.6 Seasonal anaerobic microbiology findings in the urogenital orifice of the cloaca in the Treponema infected compared to the non-Treponema infected Gilbert's potoroo population (Table 5.6, Appendix 10)

- Overall the prevalence of anaerobic species found in *Treponema* infected Gilbert's potoroos was far greater than those not infected with *Treponema*. In the *Treponema* infected population the most commonly isolated anaerobic species following *Treponema* was *Prevotella* found in 5/6 (83.3%) of trapping sessions.

- In the *Treponema* infected population, *Clostridium glycolicum* was found in four of six (66.6%) of trapping sessions and *Porphyyromonas asaccharolytica* found in three of six (50%) trapping sessions. The remaining anaerobes*, Bacteroides fragilis, Bacteroides melaninogenicus, Bacteroides thetaiotaomicron, Eubacterium lentum, Fusobacterium* and *Helococcus* sp. were only isolated in one of the six trapping sessions (16.7%).

- *Bacteroides fragilis, Bacteroides melaninogenicus, Bacteroides thetaiootaomicron, Fusobacterium* and *Helococcus* were only isolated in individuals, infected with *Treponema.*

- The highest variation in the number of species identified in the *Treponema* infected population was five species in November 2006. The highest variation in the non-*Treponema* infected population was four species in June 2006.

- In the *Treponema* infected population, the greatest number of bacterial isolates was recorded in the November 2005 trapping session (n=21), whereas the lowest number of bacterial isolates was found in June 2005 (n=11). In the non-*Treponema* infected population the greatest number of bacterial isolates was recorded in the November 2006 trapping session (n=7), the lowest number of bacterial isolates was found in June 2005 (n=0).

- SPSS® (Chicago, Illinois) univariate analysis was undertaken to determine if there was a relationship between the three most prevalent anaerobic bacteria and the presence of *Treponema* infection. The number of individuals with *Clostridium glycolicum* sp. (n = 100, Chi-Square test, p = 1), *Prevotella* sp., (n = 100, Chi-Square test, p = 0.731), *Porphyromonas asaccharolytica* sp. (n = 100, Chi-Square test, p = 0.746), and *Treponema* infection was not significantly different from the numbers of individuals infected with *Clostridium, Prevotella* and *Porphyromonas* but not *Treponema*.

Table 5.6 Seasonal urogenital anaerobic microbiology findings in the *Treponema* infected and non-*Treponema* infected Gilbert's potoroo population.

Anaerobic bacteria found in Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 14)		November 2005 (n = 20)		June 2006 (n = 11)		November 2006 (n = 13)		March 2007 (n=22)		Number of trapping sessions bacteria found	
***Treponema* Infected or non-infected**	**I**	**NI**	**I**	**NI**	**I**	**NI**	**I**	**NI**	**I**	**NI**	**I**	**NI**		
Bacteroides fragilis									1/14 7.1%				**1/6 16.7%**	**0**
Bacteroides melaninogenicus			1/13 7.7%										**1/6 16.7%**	**0**
Bacteroides thetaiotaomicron	2/11 18.2%												**1/6 16.7%**	**0**
***Bifidobacterium* sp.**												1/1 100%	**0**	**1/6 16.7%**
Clostridium glycolicum			1/13 7.7%		1/21 4.7%		2/17 11.7%	1/7 14.3%	1/14 7.1%	1/6 16.7%			**4/6 66.7%**	**2/6 33.3%**
Eubacterium lentum							1/17 5.9%	1/7 14.3%					**1/6 16.7%**	**1/6 16.7%**
Fusobacterium sp.											1/17 5.9%		**1/6 16.7%**	**0**
Helococcus	1/11 9.1%												**1/6 16.7%**	**0**
Porphyromonas asaccharolytica			1/13 7.7%		3/21 14.2%	1/1 100%		2/7 28.6%	4/14 28.6%	2/6 50%			**3/6 50%**	**3/6 50%**
Prevotella	1/11 9.1%				1/21 4.7%		3/17 17.6%	3/7 42.5%	1/14 7.1%	3/6 50%	3/17 17.6%		**5/6 83.3%**	**2/6 33.3%**
Treponema	7/11 63.6%		10/13 76.9%		16/21 76.2%		11/17 64.7%		7/14 50%		13/17 76.5%		**6/6 100%**	
Number of species in total	**4**	**0**	**4**	**0**	**4**	**1**	**4**	**4**	**5**	**3**	**3**	**1**		
Number of isolates in total	**11**	**0**	**13**	**0**	**21**	**1**	**17**	**7**	**14**	**6**	**17**	**1**		

5.3.2.7 Comparisons: seasonality, and aerobic species identified in captive and wild individuals (Table 5.7)

- Of the three most prevalent aerobes *Actinobacillus* sp*, Corynebacterium pilosum and Pasteurella* sp*. Actinobacillus* sp. was found in the wild population at a higher prevalence

in five of six trapping sessions (83.3%) compared to the captive population in four of six (66.7%) sessions.

- *Corynebacterium pilosum,* and *Pasteurella* were found in every trapping session in the wild population. In the captive population *Corynebacterium pilosum was* found in three of six trapping sessions (50%), and *Pasteurella* was found in five of six trapping sessions (83.3%).

- *Enterobacter aerogenes, Enterobacter vulneris, Lactobacillus* and *Proteus vulgaris* were found only in the captive population.

- *E. coli (*haemolytic*), Serratia marcescens, Serratia liquefaciens* and *Staphylococcus epidermidis* were only found in the wild population.

- The wild population had a greater number of species isolated at five out of six trapping sessions (83.3%).

- The wild population had a greater number of bacterial isolates at every trapping session compared to the captive population.

Table 5.7 Seasonal aerobic urogenital microbiology findings in captive compared to wild individuals.

Aerobic microbes found in Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 14)		November 2005 (n = 20)		June 2006 (n = 11)		November 2006 (n = 13)		March 2007 (n=22)		Number of sessions bacteria found	
Captive (C) or wild (W) n =	**C 4**	**W 9**	**C 6**	**W 8**	**C 6**	**W 14**	**C 4**	**W 7**	**C 4**	**W 9**	**C 5**	**W 17**	**C**	**W**
***Actinobacillus* sp.**	1/3 33.3%			1/20 5%	2/9 22.2%	9/34 26.5%		4/29 13.8%	2/11 18.2%	5/29 17.2%	4/12 33.3%	9/40 22.5%	**4/6 66.7%**	**5/6 83.3%**
***Bacillus* spp.**				1/20 5%		1/34 2.9%			1/11 9.1%	1/29 3.4%		1/40 2.5%	**1/6 16.7%**	**4/6 66.7%**
Brackiella oedipus		1/9 11.1%			1/9 11.1%	6/34 17.6%	1/5 20%	2/29 6.9%	2/11 18.2%	5/29 17.2%	3/122 5%	5/40 12.5%	**4/6 66.7%**	**5/6 83.3%**
Coliforms		1/9 11.1%			1/9 11.1%								**1/6 16.7%**	**1/6 16.7%**
Corynebacterium pilosum.		2/9 22.2%		5/20 25%		8/34 23.5%	1/5 20%	4/29 13.8%	2/11 18.2%	2/29 6.9%	1/128. 3%	3/40 7.5%	**3/6 50%**	**6/6 100%**
Enterobacter aerogenes	1/3 33.3%												**1/6 16.7%**	
Enterobacter cloacae			1/10 10%					1/29 3.4%					**1/6 16.7%**	**1/6 16.7%**
Enterobacter faecalis								2/29 6.9%		3/29 10.3%		5/40 12.5%		**3/6 50%**
Enterobacter vulneris	1/3 33.3%												**1/6 16.7%**	
E. coli (haemolytic)										1/29 3.4%				**1/6 16.7%**
E. coli (non-haemolytic)			1/10 10%	2/20 10%				2/29 6.9%		2/29 6.9%		7/40 17.5%	**1/6 16.7%**	**4/6 66.7%**
Klebsiella oxytoca			1/10 10%							3/29 10.3%			**1/6 16.7%**	**1/6 16.7%**
Lactobacillus							1/5 20%						**1/6 16.7%**	
Non-fermentive GNR			1/10 10%	4/20 20%				2/29 6.9%	2/11 18.2%				**2/6 33.3%**	**2/6 33.3%**
***Pantoea* sp.**			1/10 10%									1/40 2.5%	**1/6 16.7%**	**1/6 16.7%**
***Pasteurella* sp.**		4/9 44.4%	2/10 20%	4/20 20%	4/9 44.4%	9/34 26.5%	1/5 20%	8/29 27.6%	1/11 9.1%	4/29 13.8%	4/12 33.3%	8/40 20%	**5/6 83.3%**	**6/6 100%**
Proteus vulgaris			1/10 10%										**1/6 16.7%**	
Pseudomonas aeruginosa				1/20 5%	1/9 11.1%		1/5 20%					1/40 2.5%	**2/6 33.3%**	**2/6 33.3%**
Serratia marcescens								1/29 3.4%		1/29 3.4%				**2/6 33.3%**
Serratia liquefaciens								1/29 3.4%						**1/6 16.7%**
***Staphylococcus* sp.**		1/9 11.1%	1/10 10%					1/29 3.4%	1/11 9.1%	1/29 3.4%			**2/6 33.3%**	**3/6 50%**
Staphylococcus aureus			1/10 10%	2/20 10%		1/34 2.9%				1/29 3.4%			**1/6 16.7%**	**3/6 50%**
Staphylococcus epidermidis								1/29 3.4%						**1/6 16.7%**
Number of species in total	**3**	**5**	**9**	**8**	**5**	**6**	**5**	**12**	**7**	**12**	**4**	**9**		
Number of isolates in total	**3**	**9**	**10**	**20**	**9**	**34**	**5**	**29**	**11**	**29**	**12**	**40**		

5.3.2.8 Comparisons: seasonality, and anaerobic species identified in captive and wild individuals (Table 5.8)

- The most prevalent anaerobic species found in every trapping session, *Treponema* was found in both the captive and wild population.

- *Bacteroides melaninogenicus*, and *Bacteroides thetaiotaomicron* were found only in the captive population. *Bacteroides fragilis, Eubacterium lentum, Fusobacterium, Helococcus* and *Prevotella* were found only in the wild population.

- A greater number of bacterial isolates were found in the wild population in five of six trapping sessions (83.3%). The captive population had a greater number of bacterial isolates in one of the six trapping sessions (16.7%).

- A greater number of species were found in the wild population in five of six trapping sessions (83.3%). The captive population had a greater number of species in one of six trapping sessions (16.7%).

Table 5.8 Seasonal urogenital anaerobic microbiology findings in the captive compared to the wild population.

Anaerobic microbesfound in Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 14)		November 2005 (n = 20)		June 2006 (n = 11)		November 2006 (n = 13)		March 2007 (n=22)		Number of sessions bacteria found
Captive (C) or wild (W) population N =	**C 4**	**W 9**	**C 6**	**W 8**	**C 6**	**W 14**	**C 4**	**W 7**	**C 4**	**W 9**	**C 5**	**W 17**	
Bacteroides fragilis										1/14 7.1%			**1/6 16.7%**
Bacteroides melaninogenicus			1/7 14.3%										**1/6 16.7%**
Bacteroides thetaiotaomicron		2/6 33.3%											**1/6 16.7%**
***Bifidobacterium* sp.**												1/10 10%	**1/6 16.7%**
Clostridium glycolicum			2/7 28.6%			1/13 7.7%	1/5 20%	2/17 11.7%		1/14 7.1%			**4/6 66.7%**
Eubacterium lentum								2/17 11.7%					**1/6 16.7%**
***Fusobacterium* sp.**											1/3 33.3%		**1/6 16.7%**
Helococcus		1/6 16.7%											**1/6 16.7%**
Porphyromonas asaccharolytica			1/7 14.3%		4/8 50%			2/17 11.7%	4/6 66.7%	2/14 14.3%			**4/6 66.7%**
Prevotella		1/6 16.7%				1/13 7.7%		5/17 29.4%		5/14 35.7%		1/10 10%	**5/6 83.3%**
***Treponema* – PCR pos (+)**	2/2 100%	2/6 33.3%	3/7 42.8%	4/4 100%	4/8 50%	11/13 84.6%	4/5 80%	6/17 35.3%	2/6 33.3%	5/14 35.7%	2/3 66.6%	8/10 80%	**6/6 100%**
Number of species in total	**1**	**4**	**4**	**1**	**2**	**3**	**2**	**5**	**2**	**5**	**2**	**3**	
Number of isolates in total	**2**	**6**	**7**	**4**	**8**	**13**	**5**	**17**	**6**	**14**	**3**	**10**	

5.4 DISCUSSION

5.4.1 Results of cloacal swab of the urogenital orifice

The comparison between the urogenital findings of *Treponema* infected and non-*Treponema* infected Gilbert's potoroos is of particular interest in regards to the presence of a green tenacious urogenital discharge seen in both males and females, and captive and wild individuals. It is also interesting that the *Treponema* infected individuals had a higher prevalence of anaerobic micro-organisms than non-*Treponema* infected individuals. The reduced prevalence of *Actinobacillus* in non-*Treponema* affected individuals is also of clinical significance.

In the Gilbert's potoroo population, *Treponema* was isolated at every trapping session. *Prevotella* was the second most commonly isolated anaerobic species identified in five of the six trapping sessions (83.3%) *Porphyromonas asaccharolytica* was isolated in four of the six trapping sessions (66.7%) and *Bacteroides fragilis, Bacteroides melaninogenicus, Bacteroides thetaiotaomicron,* and *Fusobacterium* were isolated in one of the six trapping sessions (16.7%). In non-*Treponema* infected Gilbert's potoroos the most commonly isolated anaerobic species was *Porphyromonas asaccharolytica* yet this was only found in three sessions (50%). Other anaerobes such as *Bacteroides thetaiotaomicron* and *Prevotella* were only isolated in one session (16.7%). *Bacteroides fragilis, Bacteroides melaninogenicus* and *Fusobacterium* were not isolated in non-*Treponema* infected individuals. In bovine digital dermatitis (DD), microscopic examination of biopsies has revealed Gram-negative rods and large numbers of spirochaetes (Read *et al.* 1992). On culture these Gram-negative bacilli were found to be *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Porphyromonas levii* and a variety of *Prevotella* sp. (Koniarova *et al*. 1993). Therefore, the bacterial population present in conjunction with *Treponema* in DD lesions appears to be more similar to the *Treponema* infected Gilbert's potoroo population, compared to the non-*Treponema* infected population.

This may suggest that *Treponema* in Gilbert's potoroos, like treponemes involved in DD originates as a polymicrobial infection, which then leads to conditions that favour the growth of treponemes and the progression of the lesion (Edwards *et al.* 2003) This aetiology is similar to the role of *T.denticola* and other *Treponemes* involved in human periodontal disease.

In periodontal lesions, *T.denticola* is typically found in association with obligate anaerobic bacteria including *Porphyromonas gingivalis* and *Bacteroides forsythus* (Socransky *et al.* 1998). These anaerobes are thought to interact with surrounding periodontal tissues to facilitate adhesion to epithelial cells and extracellular matrix components, contribute to proteolysis and modulate host immune function to produce pathology (Lamont and Jenkinson 1998). Therefore periodontitis seems to have a polymicrobial aetiology, with enrichment for *Treponema* by preceding bacterial anaerobic population and subsequent inflammatory events concomitant with environmental changes which seem crucial to the underlying pathology.

Associated aerobic microbes also differed when comparing the *Treponema* infected population to the non-*Treponema* infected population. *Actinobacillus* was present in all trapping sessions, yet was only present in four of the six trapping sessions (66.7%) of the non-*Treponema* infected population. The *Treponema* infected population was 5.824 times more likely to have *Actinobacillus* than the non-*Treponema* infected population.

Actinobacillus is thought to be a commensal microbe of mucous membranes, therefore its presence is not surprising, however its increased prevalence in the *Treponema*-infected population may suggest localised host compromise facilitating invasion and infection. *Actinobacillus seminis* infection has been reported to cause genital disease in rams (Baynes and Simmons 1960). Al-Katib and Dennis (2007) studied four rams injected intra-epididymally with *Actinobacillus seminis.* In each ram the epididymis was enlarged and when dissected an encapsulated abscess 1 to 1.5cm in diameter with grey-yellow to greenish contents was observed. The testes of these rams were enlarged, firm and oedematous. Histologically, the

adjacent interstitium was infiltrated with mononuclear cells, a perivascular distribution of lymphocytes, plasma cells and some eosinophils were also seen. The nature of this abscess is of interest given the similarities in description to the preputial discharge seen in the Gilbert's potoroos.

In epididymitis in sheep, a polymicrobial aetiology is responsible for the pathology observed. The reduced prevalence of *Actinobacillus* in the non-*Treponema* infected population may demonstrate that *Actinobacillus* plays a role in the polymicrobial relationship, optimising conditions for the growth of *Treponema* or that factors causing reduced host compromise are present in *Treponema* infected individuals promoting *Actinobacillus* infection.

Although *Corynebacterium pilosum,* a usual commensal organism which can cause opportunistic infection, has been associated with posthitis in sheep. There was no discernible difference seen between the *Treponema*-infected individuals and non-*Treponema* infected individuals for this species. It was present in both populations at every trapping session, and the lack of significance was confirmed by the continuity correction of the Pearson chi-square (n=100, p=1).

Likewise, *Pasteurella* sp. was found in both *Treponema* infected and non-*Treponema* infected individuals at every trapping session. *Pasteurella* sp. is carried commensally on the mucous membranes of many domestic animals and disease is only seen under opportunistic conditions. When odds ratios were calculated however, the *Treponema* infected population was found to be 3.343 times more likely to have a *Pasteurella* infection than the non-*Treponema* infected population. Like *Actinobacillus,* this may reflect the role that *Pasteurella* plays in the polymicrobial relationship optimizing conditions for the growth of *Treponema,* or that factors causing reduced host compromise are present in *Treponema* infected individuals promoting *Pasteurella* infection. Interestingly, *Pasteurella* had a higher prevalence in all trapping sessions in males compared to females. In terms of the urogenital swab this may relate to the higher

potential for penis and preputial trauma during intromission, or eversion as females have no urogenital organ which can be extruded.

Escherichia coli was found in five of six trapping sessions (83.3%). When comparing the *Treponema* infected individuals to non-*Treponema* infected individuals, *E. coli* was of lower prevalence and was only found in four of six trapping sessions (66.7%). This species, typically found as a commensal in the gastrointestinal tract, is an opportunistic invader. The increased prevalence seen in the total population may reflect increased growth of the potential pathogen owing to a shift in the microbial flora as has been reported in conjunction with the *Treponema* infected population. However this altered micro-environment is still under the threshold for disease, as no clinical signs of septicaemia, respiratory or gastrointestinal disease were observed.

Brackiella oedipus was found in high prevalence in *Treponema* infected individuals in five of the six trapping sessions (83.3%). However when compared to non-*Treponema* infected individuals, *Brackiella oedipus* was of lower prevalence and was only found in four of six trapping sessions (66.7%). This identification was based on partial 16D rDNA sequencing. *Brackiella oedipus* was first isolated from the heart of a cotton-topped tamarin (*Saguinus oedipus*) undergoing a routine necropsy exam after suddenly dying during a tooth extraction procedure (Willems *et al.* 2002). No obvious signs of disease had been present, other than several bouts of diarrhoea, which is common in tamarins. This was shown to be associated with *Giardia* and *Campylobacter* which were antibiotic responsive. Necropsy findings included cardiomegaly, advanced chronic purulent epicarditis, thrombo-embolic endocarditis with mesoangioproliferative nephropathy and extramedullary haematoposeisis. The heart lesions were thought to be causally related to *Brackiella oedipus*. The high prevalence of this microbe in the urogenital tract is surprising given the paucity of information about the species and its apparent host specificity. Therefore its significance remains unknown. However, none of the Gilbert's potoroos necropsied to date have shown evidence of infection with *Brackiella oedipus*.

The most prevalent anaerobes associated with *Treponema* included *Prevotella* sp. found in five of six trapping sessions (83.3%) and *Porphyromas* sp. found in four of six trapping sessions (66.7%). However when compared to non-*Treponema* infected individuals, both *Prevotella* spp and *Porphyromonas* were of lower prevalence, found in one of six (16.7%) and three of six (50%) of trapping sessions respectively. Both commensal organisms, their lowered prevalence may correspond to the total lowered prevalence of anaerobes in the absence of *Treponema* which supports the theory that *Treponema* growth is favoured and enriched by conditions optimal for anaerobic growth. No statistically significant relationship was found between *Prevotella* sp. (n=100, p=0.731), *Porphyromonas (*n=100, p=0.746) and *Treponema* infection. Likewise *Clostridium glycolicum,* present in four of six trapping sessions (66.7%) did not have a statistically significant relationship with *Treponema* infection (n=100, p=1).

The lack of *Serratia marcescens* and *Serratia liquefaciens* in individuals which were not infected with *Treponema* is of interest, given that chronic or debilitating disease is considered a risk factor for severe infections with *Serratia* (Ania 2007). *Serratia marcescens* was isolated in two of the six trapping sessions (33.3%), while *Serratia liquefaciens* was isolated from one of the six trapping sessions (16.7%). A higher prevalence was reported in individuals with *Treponema* infection, compared to those lacking *Treponema* infection. Although *Serratia* is often associated with nosocomial infections in humans its presence in marsupials is not novel. Whittington (1988) isolated *Serratia* sp. in the platypus in one of eighteen cloacal swabs (5.5%), and in this case no clinical signs of disease were evident.

The equal and low prevalence of the commensals, *Bifidobacterium* and *Lactobacillus* (both present in one of six trapping sessions (16.7%) suggests these bacteria are colonising the urogenital tract in Gilbert's potoroos. However, it is interesting that one of the individuals which returned positive for both *Bifidobacterium* and *Lactobacillus,* was a sub-adult captive male on probiotic supplementation at the time with PROTEXIN® (Probiotics International Ltd. Stoke sub Hamdon, Somerset, UK). PROTEXIN® contains *Lactobacillus acidophilus, Lactobacillus*

delbreuckii, Lactobacillus plantarum, Lactobacillus rhamnosus, Bifidobacterium bifidum, Enterococcus faecium, and *Streptococcus salivarius*. This extra supplementation may have been acting to competitively exclude pathogens, such as *Treponema,* by inhibiting their colonisation and consequently preventing infection (Nader de Macias 1996). Alternatively it may just account for the *Bifidobacterium* and *Lactobacillus* positive culture result.

The majority of the remaining aerobes and anaerobes have been reported in other species to be commensal microbes (Appendix 7). Overall, an equal or lower prevalence for all the remaining microbes was found in the non-*Treponema* infected population as compared to *Treponema* infected population. No clinical signs of disease were evident other than green urogenital discharge seen in the majority of male *Treponema* infected individuals and some infected females.

The highest variation in the number of aerobic species identified was found in the June 2006 trapping session (winter) during which 15 species comprising 32 isolates were found.

Interestingly this is the same trapping session where the highest diversity in bacteria was found in the rectal opening within the cloaca. The highest variation in the number of anaerobic species was also found in the June 2006 trapping session during which five species comprising a total of 32 isolates were identified. When related to the prevalence of *Treponema,* the June 2006 trapping session had the second highest prevalence of *Treponema*. *Treponema* was most prevalent in the November 2005 trapping session with 80% of potoroos sampled (16/20), testing positive. Most importantly, when related to the relative proportions of anaerobic bacteria, November 2005 had the greatest number of bacterial isolates (22) from four species, supporting the theory that conditions which favour the growth of anaerobes, result in an increased prevalence of *Treponema*.

5.4.2 Results of cloacal swab of the rectal orifice

Analysis of the results of the rectal opening of the cloaca, provided no evidence of *Treponema* infection suggesting a lack of gastrointestinal colonization with *Treponema*. No clinical signs of gastrointestinal disease, including diarrhoea, malodorous, poorly formed or discoloured faeces were seen, other than in one wild male Gilbert's potoroo the cause of which seemed to be *Potostrongylus* parasitic infestation. Mixed infections with commonly reported commensal bacteria were predominantly found, including *Coryneform* bacilli*, E.coli* and *α-haemolytic Streptococcus* sp. which were found in individuals over all trapping sessions. The presence of *Coryneform* bacilli is of interest in relation to *Corynebacterium renale*. Opportunistic infections of this commensal organism include bovine pyelonephritis and ovine posthitis 'pizzle rot' (Yangawa and Honda 1978). The associated high prevalence of infection with this bacteria in the urogenital tract (found in all trapping sessions) may suggest contamination of the rectal opening from the urogenital sinus, or that *Coryneform* bacilli are commensal bacteria of the Gilbert's potoroo rectal opening of the cloaca. However, no other reports exist of *Coryneform* bacilli being isolated from the rectal opening of the cloaca in other marsupials.

Only the wild population had *Coryneform* bacilli. An explanation for this finding may be linked to the lack of captive individuals with the presumably familial renal oxalosis. The presence of renal oxalosis has been screened through urinary glycolate testing via DEC staff and the captive populations diet has been modified to minimise oxalate consumption (D.Forshaw, personal communication May, 2007). The presence of *Coryneform* bacilli in only the wild population may indicate opportunistic colonization in those predisposed to renal oxalosis.

Opportunistic urine collection for glycolate testing continues to be undertaken by DEC researchers, and it would be highly interesting to conduct further studies in this area, linking urinary glycolate levels to microbiological culture results and sediment examination. In this study no glycolate testing was undertaken, however, opportunistic free catch collection of 33 individuals

over the course of the study revealed four individuals to have urine findings consistent with a urinary tract or preputial infection (Appendix 14). Three of these four individuals (75%) were PCR positive for *Treponema* infection, had spirochetes present on dark field microscopy and had associated moderate to severe levels of discharge. Of the total population sampled, 63.6% (21/33) were PCR positive for *Treponema* infection at one point over the course of the study.

Whether these urinalysis results are reflective of urinary tract or preputial/urogenital infection is debatable. However, given that three of the four individuals were infected with *Treponema* there is a high likelihood that urogenital infection contaminated urine which was collected free catch, mid-stream. Cystocentesis would eliminate the possibility of such contamination however the invasive nature of this procedure would need to be considered. Urinalysis results revealed trends in these four cases, the pH was alkaline, proteinuria was present, WBC and RBC were elevated and the presence of bacteria in the sediment was found. The presence of opportunistic invaders, such as *Actinobacillus* sp. *Corynebacterium pilosum*, *Enterobacter*, *E. coli, Klebsiella and Prevotella* sp., on urogenital culture of these individuals is also likely to be clinically significant given bacteria were seen in the urine sediment and that comparative studies have revealed the potential pathogenicity of these species. The clinical significance of the *Brackiella oedipus* is unknown. Male GP 28 and GP 68 also had severe 3+ urogenital discharge and cloacal inflammation. Common findings amongst these two individuals were infection with *Actinobacillus* and *Treponema*. Additionally when analysing total population data (Appendix 10) from non-*Treponema* infected individuals, the lower prevalence of *Actinobacillus* in the absence of *Treponema* suggests a similar polymicrobial aetiology is responsible for the observed urogenital disease. Therefore it can be concluded that the *Treponema* flourishes when a favourable anaerobic environment exists.

In the captive and wild colony, *E. coli* was the most frequently isolated microbe found in the rectal orifice of the cloaca. The finding of *Pseudomonas* sp. exclusively in the captive population seems clinically significant. *Pseudomonas* sp. live in the soil and water and although they are found in

the faeces of normal animals (Hirsh 1979), they can rapidly over-populate when the resident bacterial microflora shifts, which often occurs in response to antimicrobial therapy. Due to its antimicrobial resistance, *Pseudomonas* will replace the normal flora and if epithelial compromise exists, colonization and subsequent infection will occur (Biberstein and Zee 1990). Although no clinical cases of *Pseudomonas* were seen throughout the study, its higher prevalence in the captive compared to the wild population may reflect a higher environmental load and so there may be the potential for infection in the event of immune compromise or during antibacterial treatment.

No evidence of *Salmonella* infection was found. However, *Salmonella* infection should always be excluded when investigating a case of gastro-intestinal disease given the potential pathogenicity of *Salmonella* in marsupial species and in particular the quokka with which the Gilbert's potoroos shares a home range.

5.5 CONCLUSION

Both male and female Gilbert's potoroos were found to have a tenacious green urogenital discharge of variable severity with associated cloacal inflammation. The presence of spirochaetes and PCR positive test results linked the presence of discharge with *Treponema* infection. Odds ratios confirmed that the *Treponema* infected population was more likely to have an associated *Actinobacillus and Pasteurella* infection than the non-infected *Treponema* population. The microbe with the greatest odds ratio when related to *Treponema* infection was *Actinobacillus*. The *Treponema* infected population was 5.824 times more likely to have *Actinobacillus* than the non-*Treponema* infected population. This may demonstrate that *Actinobacillus* plays a role in the polymicrobial relationship optimising conditions for the growth of *Treponema* or that factors causin host compromise are present in *Treponema* infected individuals promoting *Actinobacillus* infection.

Overall, the *Treponema* affected individuals had a higher prevalence of anaerobic micro-organisms based on urogenital swab results, than the non-*Treponema* infected individuals. The number of anaerobic species and the total number of isolates detected from these swabs was greater in every trapping session in the *Treponema* infected individuals compared to the non-*Treponema* infected individuals. This may suggest that *Treponema* infection in the Gilbert's potoroos, like *Treponemes* involved in bovine DD and in human periodontal disease, originates as an anaerobic polymicrobial infection, which then leads to conditions that favour the growth of *Treponema* and subsequent progression of the lesion (Edwards *et al.* 2003). Alternatively, other bacterial factors causing reduced host compromise could be present in *Treponema* infected individuals, promoting anaerobic growth and infection. These differing hypotheses will be further explored in the following chapter which outlines the findings of histopathology and culture of preputial lesions.

CHAPTER 6

The significance of a novel *Treponema* infection on the health of the Gilbert's potoroo

6.1 INTRODUCTION

The *Treponema* bacteria are a genus of Spirochaete. The other Spirochaete genera include the free-living aquatic Spirochaeta, the free-living or host-associated *Leptonema* and *Leptospira*. Spirochaetes are morphologically distinct from all other bacteria in that cells are thin and spiral shaped. All spirochaetes have one to several hundred flagella per cell encased within an outer sheath (Edwards *et al.* 2003). This allows spirochaetes to swim through highly viscous environments.

Treponemes are anaerobic and are indigenous to the mouths and genitals of humans, and to the intestinal tract and feet of animals (Edwards *et al.* 2003). The best known pathogenic member of the genus is *Treponema pallidum* (Brown *et al.* 2003). This is the agent responsible for causing syphilis, and the non-venereal endemic syphilis, yaws and pinta. Yaws and pinta are ulcerative conditions of human skin, restricted to the tropics. Endemic syphilis is a systemic disease, moist ulcerated lesions of the skin or oral or nasopharyngeal mucosa are the most common manifestations (Koff and Rosen 1993). Generalized lymphadenopathy and secondary and tertiary bone and skin lesions are also common.

In humans, *Treponema* are rarely found in healthy oral sites. Poor oral hygiene and oral health predispose to infection. Studies of sub-gingival plaque bacteria have identified high phylogenetic diversity of oral spirochaete infections, nine species of *Treponema* and 49 non-cultivated organisms showing up to a 20% difference in DNA sequence have been found (Dewhirst *et al.* 2000). Treponemes are often found in association with other microflora in active periodontitis and have been found to constitute over 50% of the total microflora in such instances (Moore *et al.* 1991). The mildest form of periodontal disease known as gingivitis occurs owing to an overgrowth of plaque with resultant inflammation. *Actinomyces* sp. *Neisseria, Haemophilus,* and *Streptococcus* predominate and Gram-negative anaerobes subsequently proliferate (Edwards *et al.* 2003). Bacteria commonly found in cases of periodontitis are seen in increasing numbers in

gingivitis. As such, gingivitis has been advocated to be the precursor to periodontitis (Tanner *et al.* 1998). Peridontitis refers to the creation of periodontal pockets by the apical advancement of plaque (Edwards *et al.* 2003). A quantitative relationship between plaque, *Treponema* load and severity of disease has been reported (Simonson *et al*. 1988). As plaque advances bacteria pathologically contact gingival tissue leading to the release of bacterial enzymes, inflammatory mediators and cytokines (Edwards *et al.* 2003). Secondary colonization with microbes such as *Fusobacterium nucleatum,* leads to local environmental changes such as low oxygen tension and provides growth factors encouraging the development of anaerobic, highly proteolytic Gram-negative organisms such as *P. gingivalis, Tannerella forsythensis* and *Treponema denticola* (Socransky *et al.* 1998). A heavy infiltration of neutrophils is typically seen in the surrounding tissues (Edwards *et al.* 2003). Without surgical and/or antimicrobial therapy inflammation and tissue destruction continues, separation of the gingival epithelium from the tooth occurs, and the tooth subsequently becomes loose, and falls out. In summary, periodontitis seems to have a polymicrobial aetiology. Environmental enrichment for *Treponema* by preceding bacterial anaerobic population and subsequent inflammatory events, concomitant with environmental change, seems crucial to the underlying pathology.

Hoof and foot diseases such as bovine digital dermatitis (DD) and contagious ovine digital dermatitis (CODD) may also share a similar polymicrobial aetiology enriched by certain environmental events from the corresponding microbial community. DD is an infectious and contagious disease often associated with lameness (Rijpkema *et al.* 1997). The disease initially presents with small, red ulcers affecting the skin on the plantar aspect of the foot between the heel bulbs. A resultant odiferous brown exudate is produced causing matting of the hair. This subsequently leads to excessive moisture, bleeding and eventual under running of the toe (Sheldon 1994). Biopsies demonstrate keratin loss, epidermal proliferation and hyperplasia with an influx of monocytes, neutrophils and lymphocytes associated with the presence of spirochaetes deep in the epidermal tissue (Edwards *et al.* 2003). The precise aetiology is still under review. However, interestingly the *Treponema* found in DD biopsies were thought to be

closely related phylogenetically to human oral *T. denticola* and *T. vincentii* (Collighan and Woodward 1997) perhaps suggestive of a similar aetiology. Recently *Treponema medium* sub-species *bovis, Treponema medium* sub-species novel, *Treponema phagedenis* sub-species novel and *Treponema pedis* subspecies. novel have been associated with bovine digital dermatitis (Evans *et al.* 2006 unpublished report).

These localised polymicrobial infections selective for Treponemes are quite distinct from the single agent of human syphilis, *Treponema pallidum*. *Treponema pallidum* is ordinarily acquired by sexual intercourse. Less common modes of transmission include nonsexual personal contact, in utero infection, and blood transfusion. *Treponema pallidum* rapidly penetrates intact mucous membranes or microscopic lesions in the skin, and within hours enters the lymphatic system and bloodstream to produce systemic infection long before the appearance of a primary lesion. The site of entry may be vaginal, rectal or oral. *Treponema pallidum* is not viable outside its host (Edwards *et al*. 2003). Incubation time is inversely proportional to the number of organisms. However, the period of time from the point of inoculation to the development of a primary lesion is usually about 4–6 weeks.

Primary syphilis clinically presents as a single, painless chancre (ulcer) at the site of infection, without treatment this will develop over the following weeks to months to secondary syphilis. This is commonly characterized by fever, lymphadenopathy, rash and genital or perineal condyloma latum (broad, flat wart like growths) (Brown *et al.* 2003). Tertiary or late syphilis is a multisystemic disease. It develops years following the initial infection and can be evidenced by neurosyphilis, generalized paresis, dementia, or cardiovascular syphilis; leading to aortic aneurysm, aortic valvular insufficiency or coronary artery ostial stenosis (Brown *et al.* 2003).

Spirochaete infections are characterized by long-term persistence of the organisms within the host, despite the usual strong immune response (Sell and Norris 1983; Steere 1989). It is the

host response, coupled with the tissue-damaging effects of bacterial factors responsible for the pathology observed in *Treponema*-associated diseases.

Over 30 species of *Treponema* have been identified. The fastidious nature of *Treponema*, together with the vast number of other bacteria present in oral and veterinary disease lesions, makes cultivation and isolation of *Treponema* difficult (Edwards *et al.* 2003). However, the advent of new isolation and culture techniques, together with molecular and immunological techniques has made it possible to classify many of these bacteria.

6.1.1 Balanoposthitis in male Gilbert's potoroos

The long history of balanoposthitis (inflammation of the penis and prepuce) and associated discharge in both the wild and captive population of the Gilbert's potoroo was discussed in Chapter 5.1.

6.1.2 *Treponema* infection in rabbits

Treponema paraluis-cuniculi has been isolated from rabbits, and is the agent responsible for 'rabbit syphilis', 'vent disease' or 'venereal spirochaetosis'. This disease has clinical parallels with the urogenital lesions observed in the Gilbert's potoroos. In rabbits, lesions are usually limited to vulval or preputial skin and begin as areas of hyperemia and oedema, then papules and vesicles develop, followed by ulceration, crusting or hyperkeratosis (Cunliffe-Beamer and Fox 1981). The lesions are reportedly painful and thought to impair breeding activity through dyspareunia (painful sexual intercourse) (Cunliffe-Beamer and Fox 1981). Lesions have also been reported on the nostrils, eyelids, mouth and base of the ears (Noguchi 1922; Adams *et al*. 1928). This is thought to represent vascular dissemination of *Treponema paraluis-cuniculi.* The occurrence of reagin antibody to *Treponema pallidum* strengthens this hypothesis (Cunliffe-Beamer 1981).

Vent disease is spread via vertical, direct and venereal contact, and carriers may be asymptomatic until stress occurs (DiGiacomo *et al*. 1983; Saito *et al.* 2003). Cunliffe-Beamer

and Fox (1981) proved through the cross-fostering of newborn rabbits that infection can occur at birth and during the suckling period. No lesions were observed in rabbits under two months of age, yet one-half of the offspring of infected does developed clinical lesions and serological titres prior to 20 weeks of age. This suggested that infection of young rabbits required prolonged contact with an infected dam or the disease had a long incubation period. Vertical transmission was thought to be capable of perpetuating venereal spirochetosis in an enzootically infected colony (Cunliffe-Beamer and Fox 1981). Therefore, infected young virgin rabbits could introduce disease into an existing breeding colony. Saito *et al.* (2003) reported the mean age of onset in household rabbits in Japan to be 8.8 months. Like Cunliffe-Beamer and Fox (1981) none of the rabbits had any mating history, and in these cases the disease was thought to be maternally transmitted. Maternally acquired infection was hypothesized to spread to the genitalia by ingestion of caecotrophs, grooming or spread from the nostrils Saito *et al.* (2003).

In adult rabbits infection has been acquired through topical contact with infected mucous membranes and venereally (Cunliffe-Beamer and Fox 1981). Lesions appeared three to five weeks following initial exposure with a positive serological test result occurring five to six weeks after lesion appearance. Cunliffe-Beamer and Fox (1981) also alluded to the possibility of rabbit-specific genetic differences increasing pathogenicity to *Treponema paraluis-cuniculi.* In particular, in-bred rabbits when inoculated with the disease developed multiple, widespread lesions of greater severity, which when later inoculated into another more genetically diverse rabbit strain produced mild, focal lesions only at the site of inoculation. This indicated dissemination was controlled by factors specific to the rabbit rather than the spirochaete.

Colony outbreaks reportedly result in decreased rates of conception, and increased rates of metritis, placenta retention, infertility and neonatal deaths (Saito *et al.* 2003). In clinical cases Saito *et al.* (2003) also commented that stress may trigger symptoms and that severe stress may be an important predisposing factor in outbreaks. Bucks may be asymptomatic carriers and carry star-shaped scars on their scrotum. The condition is definitively diagnosed via skin biopsy, silver staining (Warthrin-Starry stain), and dark field microscopy (Cunliffe-Beamer and Fox 1981). On

histopathology an ulcerative, lymphocytic dermatitis, with necrotic foci extending from the epidermis to the dermis, marked hyperkeratosis and the presence of silver stained spirochaetes has been reported (Saito *et al.* 2003). Likewise Cunliffe-Beamer and Fox (1981) reported erosions or shallow ulcers overlaid by thick crusts composed of necrotic epidermal cells and neutrophils. Epidermal microabscesses or vesicles were occasionally observed and the predominant dermal lesion involved was infiltration of plasma cells or macrophages.

The serological human Rapid Plasmid Reagin (RPR) test can be used to detect *Treponema* antibodies in rabbits, with an increasing titre post inoculation, up to 16 fold when clinical signs first appear (Saito *et al.* 2005). This test is also useful to gauge the clinical response to therapy. When positive and clinical signs are lacking, (e.g. sub-clinical or early stage infection) Saito *et al*. (2005) recommended that the individual be removed from the breeding colony due to the potential for latent infection and dispersal to other rabbits.

Rabbits with venereal spirochaetosis can be treated with parenteral penicillin, chloramphenicol and tetracyclines (DiGiacomo *et al*. 1983). Parenteral penicillin is the first choice, although it can be associated with severe digestive disturbances. Reported dose rates include penicillin G benzathine-penicillin G procaine at 42 000 to 84 000 IU/kg SC at 7 day intervals for 3 injections or penicillin G procaine at 40 000 to 60 000 IU/kg IM q24hr for 5 to 7 days (DiGiacomo *et al*. 1983). DiGiacomo *et al*. (1983) recommended that all exposed rabbits should be treated.

Cunliffe-Beamer and Fox (1981) thought eradication of the disease from enzootically infected rabbit colonies was cost effective, as genital lesions considerably reduced reproductive capabilities through dyspareunia (painful sexual intercourse). Furthermore on several occasions valuable rabbits developed severe respiratory disease due to *Pasteurella* soon after severe clinical vent disease became apparent. The authors suggested that *Treponema paraluis-cuniculi* may alter immune response, and therefore the disease should not be ignored. Similarly alterations in immune responses have been reported in rabbits experimentally infected with the closely related *Treponema pallidum* (Shell and Musher 1974).

Penicillin, is also the drug of choice in human syphilis therapy. Penicillin has been found to hasten resolution of clinical manifestations early in the course of infection; it also prevents, or, if present, halts progression of late (tertiary) stages; and prevents transmission to others (Peeling and Hook 2006). Given *T. pallidum's* relatively long generation time of 30–33 hours long-acting penicillin preparations, such as penicillin G benzathine have been the preferred option for most patients with syphilis. Penicillin allergy may prompt alternative regimes, including doxycycline and tetracycline, ceftriaxone or azithromycin. The Centers for Disease Prevention and Control (2003) recommend a single intramuscular injection of 2.4 million IU of penicillin G benzathine for treatment of early primary or secondary human syphilis. Three weekly injections of 2.4 million IU of pencillin G benzathine is the choice for late latent or tertiary (late) syphilis. While in cases of neurosyphilis aqueous crystalline penicillin G, 3 to 4 million units IV every four hours for 10 to 14 days, or penicillin G procaine 2.4 million units IM once daily, plus probenacid 500mg orally four times daily should both be prescribed for 10 to 14 days. There is currently no evidence that the organism has developed penicillin resistance (Peeling and Hook 2006).

6.1.3 Diagnosis

Dark field microscopy is the most specific technique for diagnosis of syphilis in humans where active lesions such as a chancre or condyloma latum are present (Larsen *et al.* 1995). However this test is limited by the number of live Treponemes, operator experience and the presence of non-pathogenic Treponemes, especially in oral lesions. Given this, for syphilis diagnosis in the event of a lesion, three dark field microscopy negative exams are required prior to eliminating *T. pallidum* as a causative agent (Brown *et al.* 2003).

In potoroos, antigen detection tests such as PCR and dark field microscopy provide diagnosis in the early stages of infection and are minimally invasive. PCR has been shown to have increased sensitivity compared to dark field microscopy for the detection of syphilis in humans (Morshed *et al*. 2007). This is not surprising as PCR can detect low numbers of organisms and differentiate

organisms accurately, thereby reducing the number of false-negative tests reported (Morshed *et al.* 2001).

When attempting to speciate cultivated Treponemes conventional phenotypic and genotypic characteristics can be used (Olsen *et al.* 2000). However, numerous uncultivable species also exist and through sequence analysis of 16S rRNA genes of DNA, these novel species can be classified as phylotypes. It should be noted that Treponemes have deeply branching 16S rRNA-based phylogentic groups and in some species this distance is greater than that between the families *Enterobacteriaceae* and *Pasteurellaceae* (Dewhirst *et al.* 2000). As such, Paster *et al*. (1991) has suggested that phylogenetic clustering of spirochaetal groups rather than specific percent similarity should be used to define spirochaete genera.

In humans, nine species of *Treponema* and 49 non-cultivated phylotypes showing up to a 20% difference in DNA sequence have been isolated from the oral cavity (Dewhirst *et al.* 2000). The oral Treponemes of humans fall into 10 phylogenetic groups (Paster *et al.* 2001) and 16S rRNA-based DNA probes have shown Treponemes in the oral cavity of dogs and cats to be similar to those found in the oral cavity of humans (Radolf and Lukehart. 2006) (Figure 6.1). A number of *Treponema* phylogenetic groups have also been demonstrated in DD lesions (Stamm and Trott 2006). Cultivated species and uncultivated phylotypes have been detected in these polymicrobial infections in cattle and sheep and are grouped into five phylogenetic clusters (Figure 6.2).

In humans, serological tests can also be used to demonstrate antibodies to *T. pallidum.* Infection provokes a strong humoral and cell-mediated immune response early in the course of infection (Peeling and Hook 2006). These antibodies will also remain readily detectable throughout the course of infection, and therefore are utilized to monitor the response to therapy following treatment. The resolution of both the primary and the secondary manifestations of infection

correlates with the development of cellular immune responses, both in animal models (rabbits) and in humans (Peeling and Hook 2006). Such testing has not been trialed in marsupial species.

Figure 6.1 Cultivable and non-cultivable species of *Treponema* found in the human oral cavity (Radolf and Lukehart 2006).

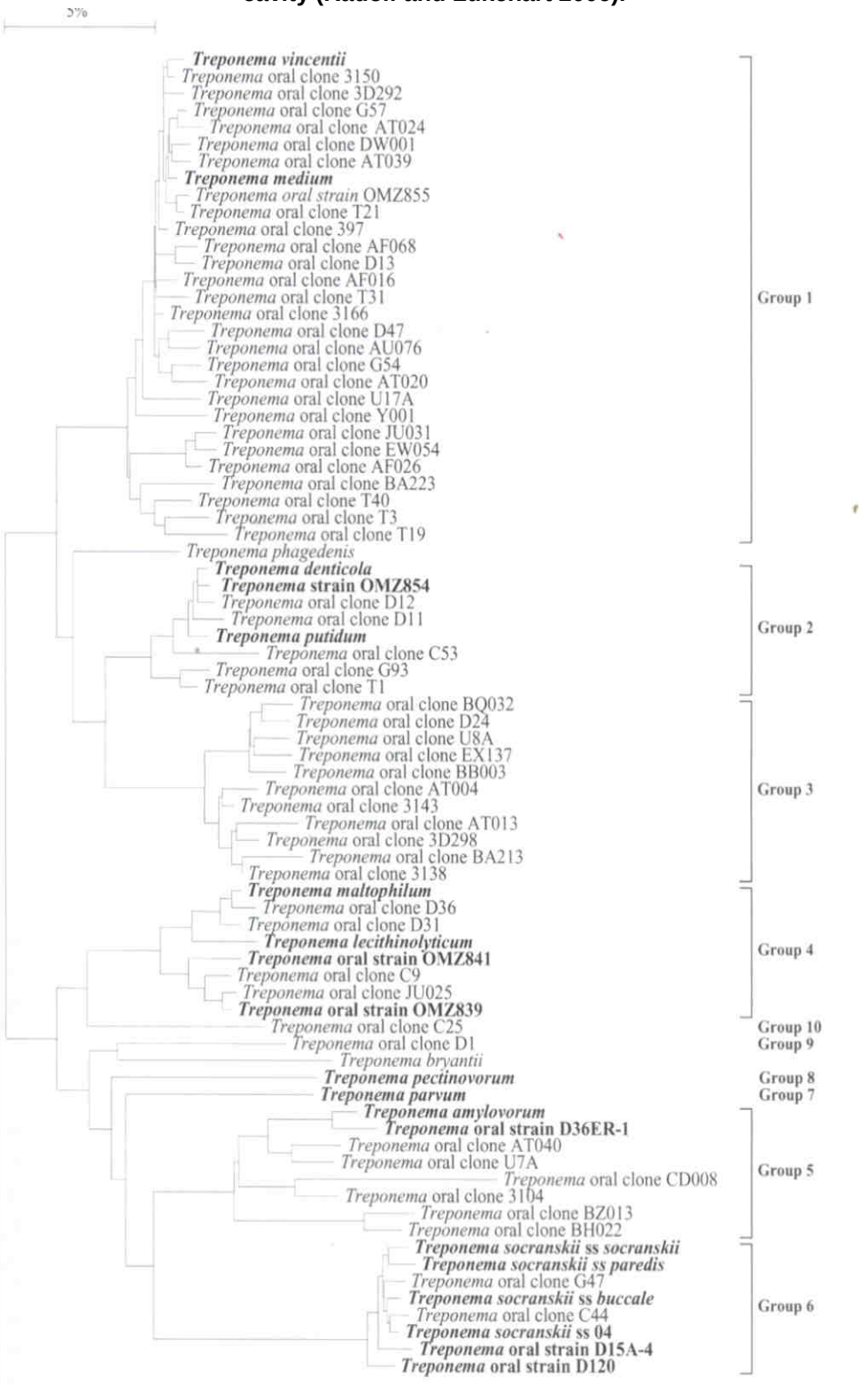
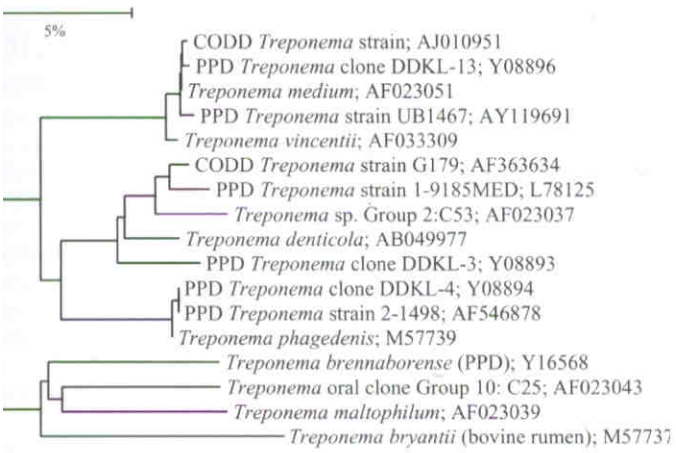


Figure 3. Phylogenetic relationships of species and uncultivated phylotypes of *Treponema* from the human oral cavity. Cultivable species are noted in bold.

Figure 6.2 Five phylotypes of *Treponema* are associated with papillomatous digital dermatitis in cattle and sheep (Radolf and Lukehart 2006).



There are a number of serological tests for *Treponema*, developed for syphilis diagnosis in humans, which also pick up antibodies to other human species. These include the *Treponema* specific tests such as the enzyme immunoassay (EIA), *Treponema pallidum* particle agglutination (TPPA), as well as the non-specific Rapid Plasmid Reagin (RPR) test. The Enzyme immunoassay (EIA) is a competitive EIA and should pick up any antibody that binds to the test antigens, whether or not it is human (D. Smith, personal communication, November 2006). The *Treponema pallidum* Particle Agglutination (TPPA) test also should work for non-human samples, but the Fluorescent Treponemal Antibody Absorption (FTA) test uses an anti-human IgG conjugate so may not be useful for testing non-human samples. It also has poor sensitivity for past infection, and has a lower specificity compared to the other *Treponema* tests.

Therefore, in searching for a serological test which could be used to screen the potoroo samples non-specific Treponema tests, such as the human RPR test may be of more clinical diagnostic value than some of the more specific *Treponema* tests, which use *T. pallidium* as the antigen in the initial screening phases. Non-treponemal tests use an antigen containing cardiolipin, cholesterol and lecithin (Nesteroff 2004). The active component in the extract is lipoidal material present in many mammalian tissues. This test detects Immunoglobulin G (IgG) and Immunoglobulin M (IgM) antibodies formed by the host in response to lipoidal material released from damaged host cells, as well as to lipoprotein-like material released from Treponemes. If the

sample is reactive it should be titreed in serial dilution and reported as reactive at the highest dilution giving a positive result. When testing humans, if this test gave a positive result, the test would then be followed by a more specific treponemal test which uses *T. pallidum* or its components as the antigen.

In Western Australia there are two tests available for quantitative evaluations of *Treponema*: the Venereal Disease Research laboratory (VDRL) test and the Rapid Plasma Reagin (RPR) test.

The VDRL test is tested on serum or CSF, while the RPR test is only run on serum. In many cases the RPR test would be adequate, as CSF sampling is highly invasive and would not be warranted in potoroos, especially given the lack of knowledge about the clinical effects of the *Treponema* organism and its unknown neurotropic status.

The potential limitations associated with use of the RPR test for potoroo samples included:

- It was unknown whether the test would work successfully on non-human samples. The RPR is a heterophile test and the relevant antigen and antibody responses may not occur in non-human species. The complexities of the marsupial immune response should also be taken into account especially given that these responses are still being further characterized (Young 2003).
- In humans, non-Treponemal tests lack sensitivity in late stage infection. It is estimated that 30% of patients with late latent or late active syphilis will show a non-reactive result (Nesteroff 2004).
- A prozone reaction has been identified in 1-2% of patients with secondary syphilis (Nesteroff 2004). This occurs when an excess of antibody in undiluted serum inhibits flocculation with the antigen. This results in weakly reactive, atypical or occasionally false negative results.
- Antibodies detected by non-Treponemal tests are produced by a number of differing conditions where tissue damage occurs. Such non-specific reactions are known as biological false positives (BFP). These false positives can occur acutely (less than six

months duration) or chronically (persisting for over six months) (Nesteroff 2004). Examples of acute biological false positives include a variety of acute, and febrile illnesses. Examples of chronic biological false positives include the autoimmune disorders such as rheumatoid arthritis and lupus and chronic infections such as leprosy, narcotic addiction, malignancy and administration of hypertensive agents. BFP's have also been reported post-immunization and occur normally in 1-2% of the general population. BFP may also be greater during pregnancy. In the case of the potoroos many of the above conditions will not apply, and by performing a thorough physical exam, CBC, biochemistry and serologically screening for specific diseases, some of these conditions will be able to be ruled out. Results from those potoroos with the presence of pouch young will need to be interpreted in light of the possibility of BFP's.

The usual method for detecting spirochaetes in tissue sections is via silver staining of formalin fixed, paraffin embedded tissue sections. However marked background staining may impede detection. Another available technique is immunohistochemistry which has the advantage of preserving tissue morphology and thus permits a detailed evaluation of the localization of spirochetes and their relationship with the surrounding tissues. The fundamental concept behind IHC is the demonstration of antigens (Ag) within tissue sections by means of specific antibodies (Abs). Once antigen–antibody (Ag-Ab) binding occurs, it is demonstrated with a coloured histochemical reaction visible by light microscopy or fluorochromes with ultraviolet light (Ramos-Vara 2005).

Hoang *et al.* (2004) compared results of immunohistochemistry (IHC) with a monoclonal antibody to *Treponema pallidum* with silver staining in 19 biopsies from 17 human patients with serologic evidence of secondary syphilis. IHC demonstrated a sensitivity of 71%, which was superior to the 41% sensitivity of the silver stain ($p = 0.084$). Furthermore, specificity was improved with IHC, as background artifacts were markedly reduced. Dermal spirochaetes were visualized in all 19

positive cases, while epidermal organisms were seen in only eight cases. Spirochaetes were not seen in any of the 14 control cases with similar histopathologic patterns

As such IHC appears more sensitive and specific than silver staining for detecting *T. pallidum* in biopsies of secondary syphilis.

6.2 MATERIALS AND METHODS

The clinical classification of urogenital lesions and discharge, and urogenital swabbing technique for *Treponema* PCR, dark field microscopy and culture have been described in Chapter 5.2.

A similar technique was carried out for disease screening purposes in five southern brown bandicoots, three yellow footed antechinus and two quokkas trapped during the course of the study. Specific methods warranting in depth explanation will be separately discussed below.

Specific molecular, serological and histopathological testing methods which were undertaken in order to investigate and identify the *Treponema* organism are outlined below. The molecular analysis of samples was primarily performed by Dr Nicky Buller of DAFWA, however I was instructed on the specific processes of PCR and sequencing to be conducted.

6.2.1 PCR

6.2.1.1 Collection and preparation of samples for PCR

The (Copan-USA) dry swab was frozen and stored at -20'C until required. DNA was extracted from material on the swab using PrepMan Ultra Sample Preparation Reagent (Applied Biosystems) as follows.

The swab was placed into one mL of sterile normal saline and agitated to release material from the swab. The resulting suspension was centrifuged for three minutes at 16 000 *x g*. The supernatant was aspirated and discarded and 200µL of PrepMan Ultra Sample Preparation

Reagent added. The sample was then vortexed for 30 seconds, heated in a thermocycler FTS 320 (Corbett) at 95 degrees for 15 minutes then centrifuged for three minutes at 16 000 *x g*. The supernatant containing the DNA was then transferred into a clean microcentrifuge tube, ready for PCR.

The primers used in the PCR for detecting a spirochaetal 16S rRNA gene are listed below. (Table 6.3) These primers were chosen following review of Rijpkema *et al.* (1997) who reported on spirochaete identification in cattle with digital dermatitis and through extensive consultation with Dr Nicky Buller, DAFWA .

Table 6.3 Primers used in the PCR for detection of a spirochaetal 16S rRNA gene.

Primer name	Location	Specificity	Sequence
EUBB	7-26	Bacteria	5'-AGAGTTGATCMTGGCTCAG
SPF1	49-68	Spirochaetes	5'-TAAGCATGCAAGTCGAGCGGGC
SPF3	336-356	Spirochaetes	5'-TCCTACGGGAGGCAGCAGCT
SPF4	833-854	Spirochaetes	5'-AGTGTACCGCCTGGGGACTATGC
SPF5	168-183	Treponaema	5'-AATACCGAATACACTCAGTGCTT
SPR1	859-835	Spirochaetes	5'-GCGGGCATACTCCCCAGGCGGYACA
SPR2	1202-1180	Spirochaetes	5'-TTGTAGCACGTGTGTAGCCCTGGA
SPR4	375-355	Treponema	5'-CCATTGCGGAATATTCTTAG
SPR5	987-965	Treponema	5'-GTACGCTGCCATATCTCTAAGTC

Primers were reconstituted in TE buffer then diluted to obtain a working concentration of 20 picomoles.

The PCR was carried out in a 25µL reaction mix using PCR Master Mix (Promega). The following reaction mix was prepared on ice in a biological cabinet to minimize cross-contamination, and amplification of non-specific products. Promega PCR master mix (12.5µL) was added to 7.5µL of sterile nuclease free water. A forward and reverse primer (1.5µL) of each was added, followed by the addition of 2µL of DNA. The following primer combinations were trialed (Table 6.4).

Table 6.4 Primer combinations trialed for detection of a spirochaetal 16S rRNA gene.

Forward primer name	Reverse primer name
EUBB	SPR4
EUBB	SPR2
SPF1	SPR4
SPF1	SPR2
SPF5	SPR2
SPF5	SPR4

The mixture was then sealed with mineral oil as the thermocycler used did not have a hot lid. PCR reactions were carried out in a thermocycler FTS 320 (Corbett). Thermocycling consisted of an initial denaturation of 94ºC for four minutes, followed by 30 cycles at 94ºC for one minute, 54ºC for one minute, 72ºC for one minute and a final cycle with an extension at 72ºC for 10 minutes.

Product was found only using SPF5 and SPR4. Therefore this combination was used as the screening test for the presence of *Treponema* infection in Gilbert's potoroos. Likewise Rijpkema *et al.* (1997) found these primers to best amplify the treponemes associated with Digital dermatitis in cattle. Asai *et al.* (2002) designed a Total Treponeme (TT), forward and reverse primer to target signature sequences unique to *T. denticola*, *T. vincentii*, or *T. medium* (Table 6.5). This Total Treponeme primer was designed to target common regions in the 16S rRNA sequences of *Treponema* species. These primers were also cross-referenced with results from Gilbert's potoroo samples in which the SPF5 and SPR4 primers were used.

Table 6.5 Total Treponeme primers cross-referenced with SPF5 and SPR4 for detection of a spirochaetal 16S rRNA gene.

Primer name	Location	Specificity	Sequence
TT		*T.denticola* *T.vincentii* *T.medium*	TTACGTGCCAGCAGCCGCGGTAAC Forward GTCRYMGGCAGTTCCGCCWGAGTC Reverse

6.2.1.3 Agarose gel electrophoresis

An agarose solution was prepared by adding 0.8g of agarose to a stock solution of 40mL of 0.5 X TBE buffer to make a 2% Agarose gel. The agar was dissolved in the microwave for 30 second

increments on high power until the liquid was boiling and the agarose dissolved. The agarose was then cooled to 60ºC and poured into a Bio-Rad gel pouring apparatus and a 30 well comb inserted into the agarose. The gel was left to set for 20 minutes at room temperature. TBE buffer was then added to the electrophoresis tank to cover the agarose up to the assigned level. Molecular weight markers were added to the first and last lanes of the gel. Each well on the gel was loaded with a combination of 5µL loading buffer (bromophenol blue dye) and 5µL of the PCR amplified product. A DNA-free negative control was also added. The gel was electrophoresed for 80 minutes at 90 volts.

The agarose gel was then removed from the electrophoresis tank and soaked in ethidium bromide to enable visualization of the dyed DNA fragments under UV light. The gel was stained for 30 minutes in a tank containing 50µL of ethidium/L of milliQ ultra-pure water. The gel image was then recorded in black and white via digital photography.

6.2.1.4 PCR Clean up

Prior to sequencing DNA was purified using an (Ultraclean) PCR Clean-Up Kit. This process removes all reaction components such as primers or linkers, enzyme, salts and dNTP's. Spinbind buffer (100µL) was added to the remaining 20µL PCR reaction. This was mixed via pipetting and two layers formed. The top layer was oil. The PCR and Spinbind mixture was then transferred to a spin filter unit, the transfer of oil being avoided. The sample was centrifuged for 30 seconds, at 10 000 *x g* in a bench-top centrifuge. The spin filter basket was then removed and the liquid flow through the tube decanted. The spin filter basket was replaced in the same tube and 300µL of SpinClean buffer added to the spin filter and centrifuged for 30 seconds at 10 000 *x g*. The spin filter basket was again removed, the liquid flow decanted and the basket replaced back into the same tube. The sample was then centrifuged for 60 seconds at 10 000 *x g* and the spin filter transferred to a clean collection tube. 50µL of Elution buffer (10nM Tris solution) was pipetted onto the center of the white spin bottle and the sample centrifuged for 60 seconds at 10 000 *x g*. The spin filter basket was then discarded with the resultant purified DNA residing in the collection tube. This DNA was stored at -20ºC and sent to Macrogen Inc. Korea, for sequencing.

When using the second batch of primers, owing to bands of non-specific amplification it was decided to load the entire remaining contents of the amplified product (approximately 20µL) onto the gel and physically remove the required band of DNA for sequencing. A gap was left between samples and the gel loaded, and electrophoresed, as described above. A photograph was not taken as the product was the same as seen previously, and minimization of UV light exposure was preferable. Using the land camera the gel was placed on a UV illuminator and individual bands were cut out using a scalpel blade and placed into eppendorf tubes for each sample. DNA was then extracted from the agarose plug using the following technique. An 18 gauge needle was used to punch a hole in the base of a 0.6mL microfuge tube. Aquarium filter wool was packed into the tube to a depth of 1cm. The 0.6mL tube was placed inside a 1.5mL microfuge tube labeled with the case number and isolate identification number. The agarose plug was placed into the labeled 0.6mL tube and the entire microfuge tube apparatus frozen in liquid nitrogen for three minutes. The tubes were centrifuged at 6000 rpm in a bench top centrifuge with the lids left open. The liquid containing the DNA passed through into the larger tube. The inner 0.6mL tube containing the wool and unwanted agarose was discarded and the amplified product cleaned up using the (Ultraclean) PCR Clean-Up Kit as above. These samples were then posted off for sequencing with the primers to Macrogen Inc, Korea.

6.2.1.5 Sequencing

All sequenced products were compared to sequence data available from GenBank, using the BLAST 2.2.14 program. Phylogenetic alignment of DNA sequences was undertaken.

6.2.2 Serology

Under anaesthesia 1mL of whole blood was collected in a plain tube and centrifuged and 0.5 µL sent to PathWest microbiology services where RPR, TPPA and the *T. pallidum* total Ab serological screening for Treponemes were undertaken. In this instance we tested serum from known PCR positive potoroos and known PCR negative potoroos which would add further validity

to the test if similar results were seen. These results were also cross-referenced with evidence of spirochaetes seen on dark field microscopy.

6.2.3 Histopathology

The biopsy procedure was conducted under general anaesthetic as part of the normal disease testing protocol in captive potoroos only. A small punch biopsy encompassing 0.3cm by 0.3cm of mucosal tissue was obtained, at an approximate tissue depth of 0.2cm. The edges of the biopsy site were apposed with an absorbable suture to dissolve over 14 days, in attempts to minimise extra handling for suture removal. A follow up check under manual restraint was performed one day following the procedure.

The tissue biopsy was placed in formalin and sent to Murdoch University via overnight courier for histological examination and silver staining for spirochaetes and any corresponding inflammatory reaction. Immunohistochemistry was also conducted on formalin-fixed, paraffin-embedded tissues.

A post mortem penile and preputial biopsy was also submitted from a long-nosed potoroo from the Perth Zoo colony. This individual had been euthanased due to severe osteomyelitis with concurrent degenerative joint disease. The biopsy was obtained using a 0.3cm punch biopsy which was placed in formalin and sent to Murdoch University via courier for histological exam and silver staining for spirochaetes and any corresponding inflammatory reaction.

6.2.4 Immunohistochemistry

In order to conduct immunohistochemistry rabbit polyclonal primary antibody to *Treponema pallidum* (ab35193) was purchased from abcam® (Cambridge, Massachusetts), since the production of monoclonal antibodies by infecting live mice was not deemed feasible as part of the study, due to the considerable cost and ongoing husbandry requirements associated with such an

experiment. Immunohistochemistry testing of formalin-fixed sections of preputial tissue which had been silver stained for spirochaetes was undertaken by Mike Slaven from the Immunohistochemisty Laboratory, Division of Pathology at Murdoch University

6.2.5 Treatment trial

A decision was made to trial the treatment regime recommended for use in rabbits, on the infected analogous long-nosed potoroos. All members of the Perth Zoo collection of long-nosed potoroos were urogenitally swabbed with a dry swab for *Treponema* PCR and a wet swab for culture and dark field microscopy as described in the materials and methods section of Chapter 5.2. Males were swabbed from the prepuce under manual restraint in accordance with Murdoch University and Perth Zoo ethical guidelines. These swabs were forwarded via courier on ice to DAFWA for microbiological analysis, culture and speciation.

All long-nosed potoroos were injected with penicillin G benzathine-penicillin G procaine at 84 000 IU/kg IM at 7 day intervals for 3 injections. Owing to the viscosity of the penicillin 18-gauge needles and 1ml syringes were used for injection into the quadriceps muscles.

Follow up swabs were taken on the day of the 3rd injection and again one month following this injection to evaluate microbiological flora and to check if spirochatetes were present under dark field microscopy and if Treponemes were present through PCR.

6.3 RESULTS

A chart grading the severity of lesions seen was formulated for ease of description for ongoing monitoring of the level of discharge and associated cloacal and preputial inflammation (Appendix 4).

Results were tabulated to compare *Treponema* infection to sex, age, origin (captive or wild), level of discharge, evidence of spirochaetes on dark field microscopy, PCR results and trapping session. *Treponema* infection was assigned based on the presence of clinical signs and associated discharge, dark field microscopy for spirochaetes and PCR results. The statistics program SPSS® (Chicago, Illinois) was used to check for statistical significance between these factors and *Treponema* infection. It should be noted that over the course of the study 44 individuals were swabbed for *Treponema*. Given the estimated current population size of the Gilbert's potoroo is approximately 35 individuals, the samples collected to assess microbiological data in relation to *Treponema* infection, were more reflective of a census rather than a population estimate, which is generally used in the majority of studies concerning statistics.

It should also be remembered that some of the following results, which are not statistically significant, may still be of clinical or biological significance. A good example is the lack of statistical significance, compared to the clinical significance of *Treponema* infection in the captive compared to the wild population. The small and static captive population has male individuals persistently infected with moderate (2+) to severe (3+) *Treponema* infections, which are then paired for mating with non-infected captive individuals which subsequently become infected. Although, there may be no statistical significance in comparisons of prevalence of infection in the captive compared to the wild population, when we have the capacity to alter the clinical consequences through selective pairing, the act of pairing a persistently infected male with a non-infected female would be of clinical significance. Therefore, the results of this study should be interpreted with due reference to clinical significance, and the ability to alter prevalence of disease in this critically endangered species. For this reason both statistically significant data analysed using SPSS® (Chicago, Illinois) and results tabulated from individual trapping sessions which are clinically significant but not statistically significant, are presented.

6.3.1 Epidemiology

6.3.1.1 Comparisons: Prevalence, level of discharge, PCR status and sex (Figure 6.6, Table 6.7, Table 6.8 and Table 6.9)

- Males showed a generalized secular trend of increased prevalence of *Treponema* infection compared to females (Figure 6.6).

- SPSS® (Chicago, Illinois) univariant analyses revealed a significant difference in susceptibility to *Treponema* infection in the male compared to the female population (n = 100, Chi-Square test, p = 0.00, OR 2.339, 95% C.I. 1.514 – 3.613). Males were 2.339 times more likely to have *Treponema* infection compared to females.

- The captive population showed a generalized secular trend of increased prevalence of *Treponema* infection compared to the wild population (Figure 6.6).

- SPSS® (Chicago, Illinois) univariant analyses revealed no significant difference in susceptibility to *Treponema* infection in the wild compared to the captive population (n = 100, Chi-Square test, p = 0.463).

- Males lacking preputial discharge did not have *Treponema* infection.

Figure 6.6 Secular trends in population prevalence of *Treponema* infection in the Gilbert's potoroo.

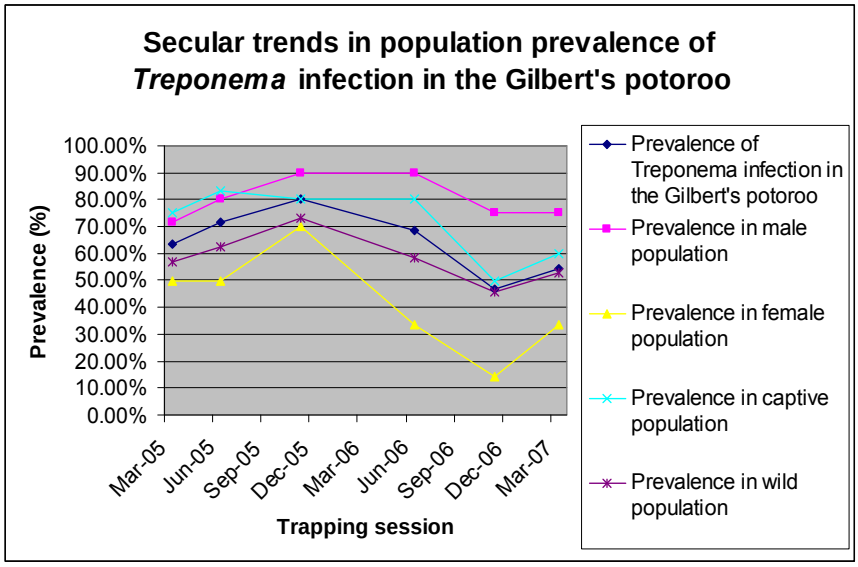


Table 6.7 Secular trends in population prevalence of *Treponema* infection in the Gilbert's potoroo.

Gilbert's potoroo population infected with *Treponema*	March 2005 (n=11)	June 2005 (n=14)	November 2005 (n=20)	June 2006 (n=16)	November 2006 (n=15)	March 2007 (n=24)
Prevalence of *Treponema* infection	63.6%	71.4%	80%	68.7%	46.7%	54.2%
Male	71.4%	80%	90%	90%	75%	75%
Female	50%	50%	70%	33.3%	14.2%	33.3%
Captive	75%	83.3%	80%	80%	50%	60%
Wild	57.1%	62.5%	73.3%	58.3%	45.5%	52.6%

- When SPSS® (Chicago, Illinois) was used to conduct a chi-square for independence to determine if there was a significant relationship between clinical signs, discharge and *Treponema* infection, a significant relationship was found (n = 100, Chi-Square test, p = 0.00, OR 29.45, 95% C.I 9.5-91.28) (Table 6.8).

- When SPSS was used to conduct a chi-square for independence to determine if there was a significant relationship between clinical signs, discharge and sex, a significant

relationship was found (n = 100, Chi-square test, p = 0.00, OR 26.8, 95% C.I 9.18-78.26) (Table 6.8).

- A generalized trend of higher percentage rates of moderate (2+) and heavy (3+) levels of discharge in males with *Treponema* infection was observed. 21 infected individuals had heavy (3+) discharge, these individuals were all male (100%). No non-infected males had 3+ discharge (Table 6.8).

- 24 individuals had moderate (2+) discharge 79.2% were *Treponema* infected (19/24), and 20.8% were non-infected (9/24) all of these individuals were male (Table 6.8 and 6.9).

- *Treponema* infected females either showed no discharge or had 1+ discharge over the course of the study (Table 6.8 and 6.9).

- Females with no discharge or mild discharge (1+) had greater percentage rates of infection with *Treponema* compared to males (Table 6.8 and 6.9).

- Mild discharge (1+) was found in 12 individuals all of whom were *Treponema* infected. Eight of these were female (66.6%) and four were male (33.3%) (Table 6.8 and 6.9).

- No discharge (0) was found in 43 individuals, 12 of which were *Treponema* infected (27.9%) and 31 were non-infected (72.1%). No males with *Treponema* infection lacked clinical signs and discharge, and 34.2% of females (12/35) with no clinical signs, and discharge had *Treponema* infection (Table 6.8 and 6.9).

Table 6.8 Cross tabulation displaying a significant relationship between *Treponema* infection, clinical signs and discharge.

			Clinical signs and discharge				Total
***Treponema* status**			**0**	**1**	**2**	**3**	**0**
	Non-infected	Count	31	0	5	0	36
		Expected Count	15.5	4.3	8.6	7.6	36.0
		% within trep	86.1%	.0%	13.9%	.0%	100.0%
		% within discharge	72.1%	.0%	20.8%	.0%	36.0%
		% of Total	31.0%	.0%	5.0%	.0%	36.0%
	Infected	Count	12	12	19	21	64
		Expected Count	27.5	7.7	15.4	13.4	64.0
		% within trep	18.8%	18.8%	29.7%	32.8%	100.0%
		% within discharge	27.9%	100.0%	79.2%	100.0%	64.0%
		% of Total	12.0%	12.0%	19.0%	21.0%	64.0%
Total		Count	43	12	24	21	100
		Expected Count	43.0	12.0	24.0	21.0	100.0
		% within trep	43.0%	12.0%	24.0%	21.0%	100.0%
		% within discharge	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	43.0%	12.0%	24.0%	21.0%	100.0%

Table 6.9 Cross tabulation displaying a significant relationship between clinical signs and discharge, and sex.

			Clinical signs and discharge				Total
			0	**1**	**2**	**3**	**0**
Sex	**Male**	Count	8	4	24	21	57
		Expected Count	24.5	6.8	13.7	12.0	57.0
		% within sex	14.0%	7.0%	42.1%	36.8%	100.0%
		% within discharge	18.6%	33.3%	100.0%	100.0%	57.0%
		% of Total	8.0%	4.0%	24.0%	21.0%	57.0%
	Female	Count	35	8	0	0	43
		Expected Count	18.5	5.2	10.3	9.0	43.0
		% within sex	81.4%	18.6%	.0%	.0%	100.0%
		% within discharge	81.4%	66.7%	.0%	.0%	43.0%
		% of Total	35.0%	8.0%	.0%	.0%	43.0%
Total		Count	43	12	24	21	100
		Expected Count	43.0	12.0	24.0	21.0	100.0
		% within sex	43.0%	12.0%	24.0%	21.0%	100.0%
		% within discharge	100.0%	100.0%	100.0%	100.0%	100.0%
		% of Total	43.0%	12.0%	24.0%	21.0%	100.0%

6.3.1.2 Comparisons: Treponema, Pasteurella and Actinobacillus infection in relation to clinical signs and presence of discharge

- In Chapter 5 we ascertained that the two microbes most commonly found in association with *Treponema* were *Actinobacillus* and *Pasteurella*.

- When these bacteria were examined individually we found that individuals with *Treponema* infection were 26.87 times more likely to have the presence of discharge than the non-*Treponema* infected population (n = 100, Chi-Square test, p = 0.00, OR 26.87 95% C.I 8.64-83.51).

- Individuals with *Actinobacillus* infection were 5.33 times more likely to have the presence of discharge than the non-*Actinobacillus* infected population (n = 100, Chi-Square test, p = 0.001, OR 5.33 95% C.I 2.04-13.94).

- Individuals with *Pasteurella* infection were 4.63 times more likely to have the presence of discharge than the non-*Pasteurella* infected population (n = 100, Chi-Square test, p = 0.00, OR 4.63 95% C.I 1.94-11.03).

- Therefore the presence of discharge was positively associated with the presence of all three microbes. However individuals with *Treponema* infection were most likely to have the presence of discharge (OR 26.87 95% C.I 8.64-83.51) followed by those with *Actinobacillus* (OR 5.33 95% C.I 2.04-13.94) and then *Pasteurella* (OR 4.63 95% C.I 1.94-11.03).

- Individuals with discharge were 19.73 times more likely to have *Pasteurella* infection than those without *Treponema* infection (n = 100, Chi-Square test, p = 0.00, OR 19.73 95% C.I 6.17-63.08). Likewise individuals with *Actinobacillus* infection were 25.69 times more

likely to have the presence of discharge than the non-*Treponema* infected population (n = 100, Chi-Square test, p = 0.00, OR 25.69 95% C.I 7.33-90.05).

- When the presence of discharge was compared to those individuals without *Treponema*, *Pasteurella* and *Actinobacillus* infection, no statistically significant values were found.

- As such the presence of discharge is associated with a polymicrobial environment containing *Treponema*, *Actinobacillus* and *Pasteurella*. *Treponema* infection is most positively associated with the presence of discharge, however those lacking *Treponema* infection with *Actinobacillus* infection (OR 25.69 95% C.I 7.33-90.05) and/or *Pasteurella* infection (OR 19.73 95% C.I 6.17-63.08) still presented with discharge.

6.3.1.3 Comparisons: seasonality, Treponema infection and sex (Table 6.7 and Table 6.10)

- The highest prevalence of *Treponema* infected Gilberts potoroos (both male and female, captive and wild) was found in the November (spring) 2005 trapping session with 16 of 20 (80%) individuals positive for the infection (Table 6.7).

- In the March (autumn) trapping session of 2005, 63.6% of Gilberts potoroos (7/11) were *Treponema* infected as compared to the March 2007 trapping session where 54.2% were *Treponema* infected (13/24). The prolonged courier service and potential lack of suitable product should be taken into account when interpreting the data from the March 2007 session.

- In the June (winter) trapping session of 2005, 71.4% of Gilberts potoroos (10/14) were *Treponema* infected as compared to the June 2006 trapping session where 68.7% were *Treponema* infected (11/16).

- In the November (spring) trapping session of 2005, 80% of Gilberts potoroos (16/20) were *Treponema* infected as compared to the November 2006 trapping session where, 46.7% were *Treponema* infected (7/15).

- A one-way between groups analysis of variance using SPSS® (Chicago, Illinois) was conducted to explore the prevalence of *Treponema* infection over the six trapping sessions. No statistically significant difference in *Treponema* infection over the six trapping sessions ($p = 0.349$) was found.

- The combined prevalence for the autumn, winter and spring trapping sessions was 20/35 (57.1%), 21/30 (70%) and 23/35 (65.7%) respectively. Therefore, the highest prevalence of infection was seen over the June (winter) trapping sessions 21/30 (70%).

- A one-way between groups ANOVA with planned comparisons was conducted to explore the prevalence of *Treponema* infection over the June (winter) trapping sessions compared to the March (autumn) and November (spring) trapping sessions. No statistically significant difference in *Treponema* infection over the June (winter) season, compared to March (autumn) or November (spring) was found ($F (1, 53.502) = 0.714$, $p = 0.402$).

Table 6.10 Seasonal *Treponema* prevalence, presence and level of discharge in the captive compared to the wild population.

Gilbert's potoroos		March 2005 (n = 11)	June 2005 (n = 14)	November 2005 (n = 20)	June 2006 (n = 16)	November 2006 (n = 15)	March 2007 (n = 24)
Captive Population		**(n = 4)**	**(n = 6)**	**(n = 5)**	**(n = 5)**	**(n = 4)**	**(n = 5)**
***Treponema* infected TOTAL**		3/4 75%	5/6 83.3%	4/5 80%	4/5 80%	2/4 50%	3/5 60%
Level of discharge in *Treponema* infected individuals	**0**		1/5 20%	1/4 25%		1/2 50%	
	1+	1/3 33.3%	1/5 20%		2/4 50%		1/3 33.3%
	2+	1/3 33.3%				1/2 50%	1/3 33.3%
	3+	1/3 33.3%	3/5 60%	3/4 75%	2/4 50%		1/3 33.3%
Wild Population		**(n = 7)**	**(n = 8)**	**(n = 15)**	**(n = 12)**	**(n = 11)**	**(n = 19)**
***Treponema* infected TOTAL**		4/7 57.1%	5/8 62.5%	11/15 73.3%	7/12 58.3%	5/11 45.5%	10/19 52.6%
Level of discharge in *Treponema* infected individuals	**0**	1/4 25%		6/11 54.5%			
	1+		1/5 20%		2/7 28.57%		3/10 30%
	2+	2/4 50%	3/5 60%	2/11 18.2%	1/7 14.3%		3/10 30%
	3+	1/4 25%	1/5 20%	3/11 27.3%	4/7 57.1%	5/5 100%	4/10 40%

6.3.1.4 Comparisons: clinical signs and level of discharge, Treponema infection, and origin (Table 6.10, and 6.11)

- The captive population had a higher prevalence of severe (3+) discharge in *Treponema* infected individuals compared to the wild population during the first three trapping sessions March 2005 (33.3%), June 2005 (60%) and November 2005 (75%) (Table 6.8).

- The wild population had a higher prevalence of severe (3+) discharge in *Treponema* infected individuals during the June 2006 (57.14%), November 2006 (100%) and March 2007 (40%) trapping session. However no severe (3+) discharge was evident in captive *Treponema* infected individuals during the November 2006 trapping period (Table 6.10).

- The wild population had a higher prevalence of *Treponema* infected individuals lacking discharge compared to the captive population over all trapping sessions. All of the individuals which were *Treponema* infected and lacked discharge were females (Table 6.10).

- SPSS® (Chicago, Illinois) univariate analysis was undertaken to determine if there was a relationship between the clinical signs, level of discharge and origin (wild or captive individuals). No statistical significance was found (n = 100, Chi-Square test, p = 0.226) (Table 6.11).

Table 6.11 Cross tabulation displaying a lack of statistical association between origin and clinical signs and level of discharge.

		Clinical signs and level of discharge				Total
		0	**1**	**2**	**3**	
Wild	Count	34	6	18	14	72
	Expected Count	31.0	8.6	17.3	15.1	72.0
	% within origin	47.2%	8.3%	25.0%	19.4%	100.0%
	% within discharge	79.1%	50.0%	75.0%	66.7%	72.0%
	% of Total	34.0%	6.0%	18.0%	14.0%	72.0%
Captive	Count	9	6	6	7	28
	Expected Count	12.0	3.4	6.7	5.9	28.0
	% within origin	32.1%	21.4%	21.4%	25.0%	100.0%
	% within discharge	20.9%	50.0%	25.0%	33.3%	28.0%
	% of Total	9.0%	6.0%	6.0%	7.0%	28.0%
Total	Count	43	12	24	21	100
	Expected Count	43.0	12.0	24.0	21.0	100.0
	% within origin	43.0%	12.0%	24.0%	21.0%	100.0%
	% within discharge	100.0%	100.0%	100.0%	100.0%	100.0%
	% of Total	43.0%	12.0%	24.0%	21.0%	100.0%

6.3.1.5 Comparisons: Treponema infection, clinical signs and level of discharge, sex, and dark field microscopy for spirochaetes (Table 6.12, 6.13, 6.14, 6.15, 6.16, 6.17 and 6.18).

- Spirochaetes were present in all individuals with *Treponema* infection (Table 6.12).

- When SPSS® (Chicago, Illinois) was used to conduct a chi-square for independence to determine if there was a significant relationship between the level of spirochaetes and *Treponema* infection, a significant relationship was found (n = 100, Chi-Square test, p = 0.00) (Table 6.11). An odds ratio was unable to be calculated as all individuals with *Treponema* infection had spirochaetes.

- When SPSS® (Chicago, Illinois) was used to conduct a chi-square for independence to determine if there was a significant relationship between PCR results and *Treponema* infection, a significant relationship was found (n = 100, Chi-Square test, p = 0.00, OR 4.54, C.I. 2.37 – 8.67) (Table 6.14).

Table 6.12 Seasonal *Treponema* prevalence and level of spirochaetes found on dark field microscopy.

Gilbert's potoroos		March 2005		June 2005		November 2005		June 2006		November 2006		March 2007	
		(n = 11)		(n = 14)		(n = 20)		(n = 16)		(n = 15)		(n = 24)	
***Treponema* infected TOTAL**		7/11 (63.6%)		10/14 (71.4%)		16/20 (80%)		11/16 (68.7%)		7/15 (46.6%)		13/24 (54.2%)	
***Treponema* infected showing discharge**		¾ 75%		9/10 90%		10/16 62.5%		11/11 100%		6/7 85.7%		13/13 100%	
Sex affected		**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Level of discharge in *Treponema* infected potoroos	**0**		1	1	3		6				1		
	1+			1	1	1	1	2	2				4
	2+	1	1	4		4		1		4		5	
	3+	1		4		4		6		2		4	
Level of spirochaetes seen on DFM in *Treponema* infected potoroos	**0**	5/7 71.4%	2/7 28.6%	6/10 60%	3/10 30%	3/16 18.7%	3/16 18.7%					2/13 15.4%	2/13 15.4%
	1+					2/16 12.5%	4/16 25%						1/13 7.7%
	2+					3/16 18.75%						5/13 30%)	1/13 7.7%
	3+			1/10 10%		1/16 6.25%						2/13 15.4%	
Treponema infected & spirochaetes on DFM		0/7% 0%		1/7 14.3%		10/16 62.5%						9/13 69.2%	
Treponema infected & no spirochaetes seen on DFM		7/7 100%		6/7 85.7%		5/16 21.2%						4/13 30.7	

Table 6.13 Cross tabulation displaying a significant relationship between *Treponema* infection and the presence of spirochaetes seen under dark field microscopy.

		Spirochaetes			Total
		1	**2**	**3**	
***Treponema* infected**	Count	5	15	4	24
	Expected Count	5.0	15.0	4.0	24.0
	% within trep	20.8%	62.5%	16.7%	100.0%
	% within spirochetes	100.0%	100.0%	100.0%	100.0%
	% of Total	20.8%	62.5%	16.7%	100.0%
Total	Count	5	15	4	24
	Expected Count	5.0	15.0	4.0	24.0
	% within trep	20.8%	62.5%	16.7%	100.0%
	% within spirochetes	100.0%	100.0%	100.0%	100.0%
	% of Total	20.8%	62.5%	16.7%	100.0%

Table 6.14 Cross tabulation displaying a significant relationship between *Treponema* infection and PCR positive results.

		PCR results		Total
***Treponema* status**		**Negative when positive**	**Positive when positive**	
Non-infected	Count	36	0	36
	Expected Count	14.0	22.0	36.0
	% within trep	100.0%	.0%	100.0%
	% within Trep neg	92.3%	.0%	36.0%
	% of Total	36.0%	.0%	36.0%
Infected	Count	3	61	64
	Expected Count	25.0	39.0	64.0
	% within trep	4.7%	95.3%	100.0%
	% within trep neg	7.7%	100.0%	64.0%
	% of Total	3.0%	61.0%	64.0%
Total	Count	39	61	100
	Expected Count	39.0	61.0	100.0
	% within trep	39.0%	61.0%	100.0%
	% within trep neg	100.0%	100.0%	100.0%
	% of Total	39.0%	61.0%	100.0%

- All individuals that had spirochaetes on dark field microscopy had *Treponema* infection (100%). However, not all individuals with clinical signs and discharge had *Treponema* infection, 5 males with moderate (2+) discharge did not have *Treponema* infection and 12 females with no discharge had *Treponema* infection. Additionally 3 individuals were PCR negative despite having 3+ discharge and the presence of spirochaetes on dark field microscopy. Therefore, clinical signs and the presence of discharge and PCR positivity is not as sensitive or specific as the finding of spirochaetes on dark field microscopy. A two-by-two table was used to evaluate each individual tests sensitivity and specificity (Table 6.15).

Table 6.15 Sensitivity and specificity of diagnostic tests

	Diseased	Not diseased
Test positive	A	C
Test negative	B	D

- Sensitivity refers to the probability of a positive test amongst patients with disease = a/(a+c) and specificity is the probability of a negative test amongst patients without disease = b(b+d). The sensitivity of diagnosing *Treponema* infection through clinical signs and discharge is 52/57 (91.2%). While the specificity of diagnosing *Treponema* infection through clinical signs and discharge is 12/43 (27.9%). (Table 6.16). The sensitivity of diagnosing *Treponema* infection through dark field microscopy for spirochetes is 24/24 (100%). The specificity of diagnosing *Treponema* infection through dark field microscopy for spirochaetes was unable to be calculated as there were no individuals sampled for DFM that lacked *Treponema* infection (Table 6.17).

Table 6.16 Sensitivity and specificity of diagnosing *Treponema* infection through clinical signs and discharge.

Treponema infection and clinical signs and discharge	Diseased	Not diseased
Test positive	52	5
Test negative	12	31

Table 6.17 Sensitivity and specificity of diagnosing *Treponema* infection through dark field microscopy for spirochaetes.

Treponema infection and dark field microscopy for spirochaetes	Diseased	Not diseased
Test positive	24	0
Test negative	0	0

- The sensitivity of diagnosing *Treponema* infection through PCR is 61/64 (95.3%). While the specificity of diagnosing *Treponema* infection through PCR is 36/36 (100%). (Table 6.18). Therefore, of the three methods of diagnosis: 1.) clinical signs and level of discharge, 2.) dark field microscopy for spirochaetes and 3.) PCR, the most sensitive test

to conduct was dark field microscopy for spirochaetes and the most specific test conducted was PCR.

Table 6.18 Sensitivity and specificity of diagnosing *Treponema* infection through PCR.

Treponema infection and PCR results	Diseased	Not diseased
Test positive	61	0
Test negative	3	36

- The differing sample sizes should be remembered when evaluating this data as there were only 24 samples of 100 which had dark field microscopy undertaken as compared to 100 samples from which PCR and a clinical comment on the nature of the discharge was made.

- The discrepancy in numbers between samples collected for dark field microscopy and clinical comments on discharge is owing to logistical constraints in accessing a dark field microscope within 24 hours from the field site in the March, June 2005 and June 2006 trapping sessions.

6.3.1.6 Comparisons: age and PCR status (Table 6.19)

- Male and female, adult and juvenile potoroos from the wild and captive populations are infected with *Treponema*.

- SPSS® (Chicago, Illinois) univariate analyses revealed a significant difference in susceptibility to *Treponema* infection in the adult compared to the sub-adult population (n = 100, Chi-Square test, p = 0.00, OR 6.044, 95% C.I. 2.434 – 15.012). Adults were 6.044 times more likely to have *Treponema* infection compared to sub-adults.

- Adult females had a higher prevalence of *Treponema* infection compared to adult males in the March 2005 (66.7%), June 2005 (66.7%) and the November 2005 (100%) trapping sessions.

- Adult females had a higher prevalence of PCR positive results for *Treponema* infection compared to adult males in the March 2005 (100%), June 2005 (100%) and November 2005 (100%) trapping sessions.

- Adult males had a higher prevalence of *Treponema* infection compared to adult females in the June 2006 (100%) November 2006 (100%) and March 2007 (100%) trapping sessions.

- Male juveniles compared to female juveniles had an increased or equal prevalence of *Treponema* infection, over the course of the study.

6.3.1.7 Comparisons: presence of pouch young in Treponema infected females (Table 6.19)

- Females with *Treponema* infection appeared to still be reproducing in November 2005. At this time four adult females with pouch young (F50, F69, F89 and F100), showed no evidence of urogenital discharge, yet had low numbers of spirochaetes seen under dark field microscopy and were PCR positive for the *Treponema* infection.

- The above four females were not re-trapped in June 2006. In November 2006, F50 was negative as was F69 who also had a pouch young at this time. F89 and F100 were not re-trapped at this session. In March 2007, only F100, a young adult, remained *Treponema* infected and lacked pouch young. This individual also had evidence of 1+ green urogenital discharge, and low numbers of spirochaetes on dark field microscopy. At this time the other two females, (F69 and F89) were re-trapped and had no evidence of discharge or evidence of spirochaetes on dark field microscopy. Both had the presence of pouch young.

- In March 2007 *Treponema* infected F100 had no pouch young while non-*Treponema* infected F69 and F89 both had pouch young.

Table 6.19 *Treponema* infection prevalence in various age categories and in females with the presence of pouch young.

Gilbert's potoroos		March 2005 (n = 11)		June 2005 (n = 14)		November 2005 (n = 20)		June 2006 (n = 16)		November 2006 (n = 15)		March 2007 (n = 24)	
Gender		**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**
Age Category	**SA**	1	1	1	1	1	1	4	1	2	2	4	4
	A	7	2	10	2	10	8	6	5	5	6	8	8
% of *Treponema* infected individuals in age category	**SA**							3/4 75%		1/2 50%		1/4 25%	1/4 25%
	A	5/7 71.4%	2/2 100%	8/10 80%	2/2 100%	8/10 80%	8/8 100%	6/6 100%	2/5 40%	5/5 100%	1/6 16.7%	8/8 100%	3/8 37.5%
***Treponema* infected TOTAL**		7/11 63.6%		10/14 71.4%		16/20 80%		11/16 68.8%		7/15 46.7%		13/24 54.2%	
***Treponema* infected females with pouch young**		0%		0%		4/8 50%		0%		0%		0%	

***SA** refers to a sub-adult individual under 800g, which is not sexually mature: **A** refers to an adult.

6.3.1.8 Comparisons: seasonality, Treponema infection and con-specific species (Appendix 11)

- Over the course of the study 44 Gilbert's potoroos were sampled. Two females (one wild and one captive cleared the infection). Clearance was defined as two consecutive PCR negative (-) results. Three other females (F50, F78 and F89) had one PCR negative (-) result following infection, but did not have a subsequent sample collected due to the length of the study or the lack of trapping success, and since the subsequent second negative status was unable to be confirmed these individuals were not classified as having 'cleared infection'.

- Seven males and one female, once infected, became persistently infected. Classification as persistently infected was defined by three consecutive PCR positive (+) results.

- Two individuals were swabbed throughout the trial and had an overall negative (-) status. An overall negative status was defined by two consecutive PCR negative (-) results indicating absence of infection with *Treponema*. These were adult GP F86 and adult GP M98.

- No clinical signs, urogenital discharge or evidence of *Treponema* infection was found using dark field microscopy for spirochaetes or through urogenital swabs for *Treponema* PCR in five southern brown bandicoots, three yellow footed antechinus and two quokkas.

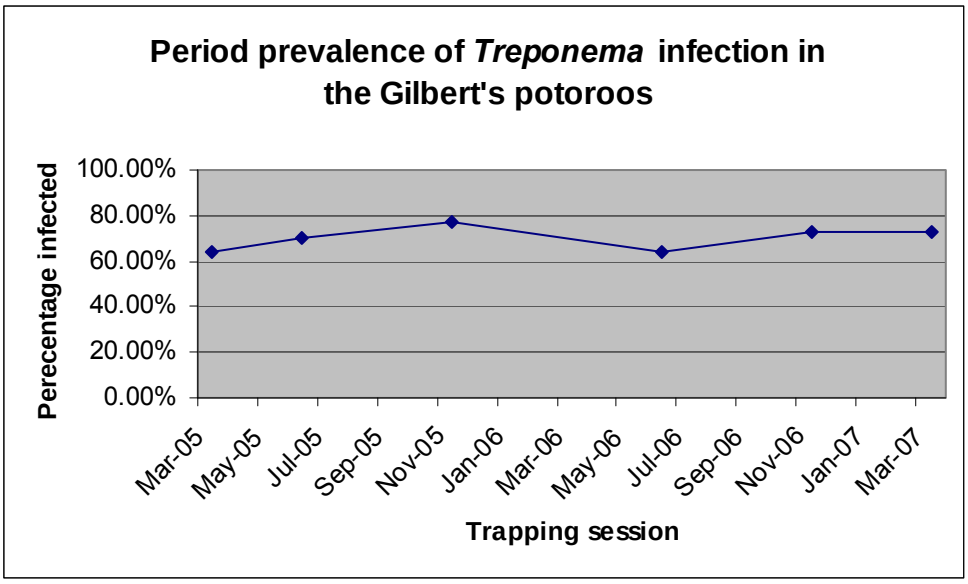
6.3.1.9 Period prevalence and total population prevalence of Treponema throughout study (Figure 6.20)

- 32/44 individuals were positive for *Treponema* infection over the course of the study.

- The period prevalence of *Treponema* infection, was calculated by adding new infected cases to existing infected cases. However, given the fact that two initially infected females cleared infection these individuals were no longer classed as infected and so the true period prevalence was corrected to be 68.2% (30/44).

- It should also be noted that during the final trapping session the courier service was prolonged and the samples were not delivered within the specified 18 hours leading to thawing and loss of biological activity. This may have artificially lowered the true prevalence of infection.

Figure 6.20 Period prevalence of *Treponema* infection in the Gilbert's potoroo.



6.3.2 PCR

Given the novel nature of this work, investigation was undertaken in collaboration with Nicky Buller from the DAFWA to find suitable primers to amplify the DNA. Many primers were trialed with no success, yet success was found using the SPF5 and SPR4 primers.

Both types of swabs (wet and dry) were also compared for DNA amplification. Agar contains inhibitors for the PCR reaction, therefore there was less amplified product (a smaller band) seen in the gel from the 'wet swab'.

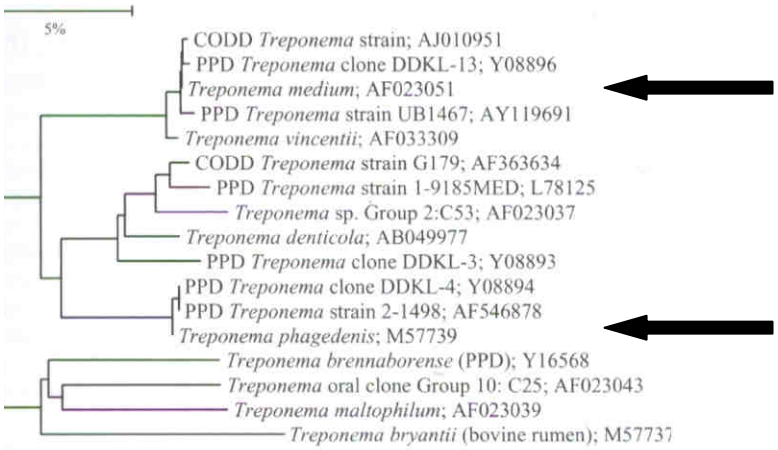
When product sequenced from the SPF5 and SPR4 primers was identified using the BlastN against the sequence information in Genbank a small sequence of approximately 190 base pairs revealed a 92% maximal identity to an uncultured *Treponema* clone. The results gave a number of similarities: there were environmental samples, 10 hits from 5 organisms: *Treponema phagedensis*, 15 hits from 2 organisms and *Treponema phagedenis* subsp. *vaccae* 14 hits from 1 organism (equivalent name *Treponema bovis*). This taxonomic name was not validly published at

the time of submission of the corresponding sequence entry (Evans *et al.* 2006 unpublished report).

These results were similar to the preliminary phylogenetic study conducted in 2002. Product from the original sequencing conducted in 2002 (amplified using the universal primers SPF5 and SPR4) was also re-blasted and was identified as having a 92% maximal identity to an uncultured *Treponema* clone. Using the BlastN against the sequence information in Genbank similarities to *Treponema medium* subsp. *bovis*, *Treponema medium* genomic RNA for 16S ribosomal RNA and *Treponema* sp. oral clone JUO31 were found. None of these species were able to be cultured.

Therefore it appears from partial 16S sequencing that the *Treponema* infection found in the Gilbert's potoroo is most similar to the *Treponema* phylotypes associated with papillomatous digital dermatitis found in cattle and sheep. The two groups most similar to the sequence results found in Gilbert's potoroo are group one, which appear to closely cluster with oral species *T. medium/vincentii* and group 3 which is identical to *T. phagedenis*.

Figure 6.21 ***Treponema*** **infection in Gilbert's potoroos is most similiar to phylotypes of** ***Treponema*** **associated with papillomatous digital dermatitis in cattle and sheep (Radolf and Lukehart 2006).**



Due to non-specific amplification, alternative primers were also trialed. Asai *et al.* (2002) Total Treponeme (TT), forward and reverse primers, designed to target common regions in the 16S rRNA sequences of *Treponema* species produced product. However, when results from these primers were cross-referenced with SPF5 and SPR4 results, differences were found suggesting that multiple Treponemes may be involved in the pathogenesis of balanoposthitis in the Gilbert's potoroo. Tracing back through the results there were five individuals that were TT positive, and SPF5 and SPR4 negative. The results from sequencing the product of the forward and reverse primers had a 98% maximum identity with uncultured Porphytomonadacea clone. A genus within this Family is *Porphyromonas* (previously *Bacteroides*) (Chapter 5.1.3.14). It was thought that the TT primers were cross-reacting with *Porphyromonas* sp. This theory was tested by performing PCR using the TT primers against a *Bacteroides* species and a *Clostridium* species isolated from a Gilbert's potoroo. Amplified product was obtained from bands at 150, 450 and 650 bp. Product from the TT primers tested using swab material produced a strong band of product at 650 bp.

6.3.3 Serology

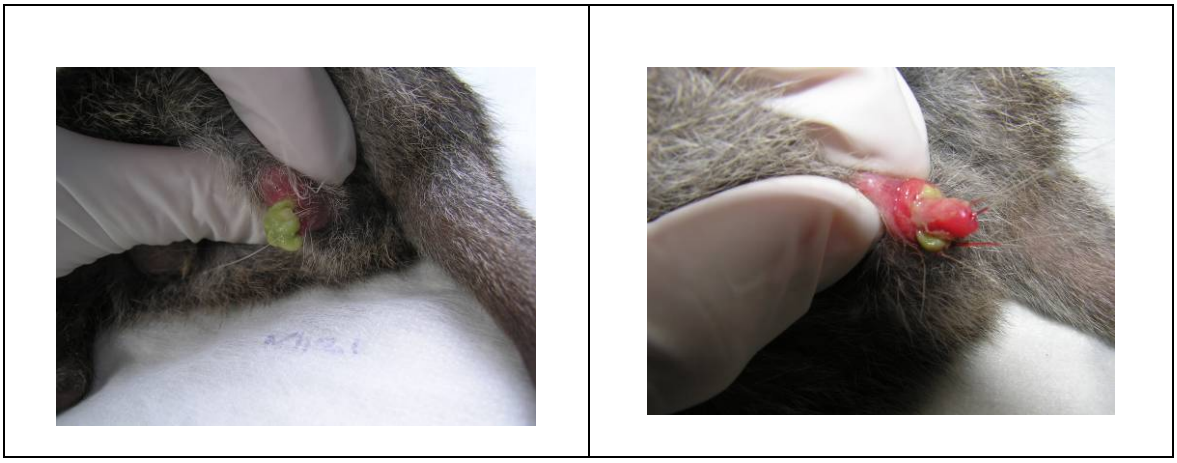
Serum was collected to detect any antibody response to any of our known PCR positive Gilbert's potoroos in the March 2007 trapping session. These samples were screened for anti-Treponeme antibodies using the RPR, TPPA and the *T. pallidum* total Ab. No antibody response was detected in any of the 15 samples submitted. Eight of these samples were PCR positive for *Treponema* infection.

6.3.4 Histopathology and Immunohistochemistry

Tissue biopsy for culture, Warthrin-Starry silver staining, and histopathology was conducted in the November 2006 trapping session on two captive males. These two males had severe (3+) preputial discharge, cloacal and preputial inflammation and ulceration (Figure 6.22).

The preputial biopsy site when examined one day post-biopsy procedure appeared clean and intact, and minimal scarring was evident when re-checked 3 months following the procedure.

Figure 6.22 Severe balanoposthitis in two male captive Gilbert's potoroos.



Tissue biopsies were examined histologically for spirochaetes and any corresponding inflammatory reaction. A moderate chronic erosive inflammatory response with diffuse moderate to marked secondary epithelial hyperplasia, in conjunction with moderate numbers of spirochaetes was found, suggesting a strong causative relationship (Figure 6.23). The silver stained spirochaetes 2-4µm in length, were diffusely scattered over the mucosal surface and extended into the lamina propria in association with a moderate accumulation of inflammatory cells, predominantly macrophages, and lesser numbers of lymphocytes and plasma cells (Figure 6.24). A moderate diffuse intracellular swelling of epithelial cells was evident along the prepuce. Culture revealed a heavy mixed growth of the *Actinobacillus-Pasteurella* group and *E.coli*. These were thought to be commensal organisms or secondary opportunistic organisms. A second biopsy reported similar findings in conjunction with a low to moderate number of neutrophils forming small microabscesses within the epithelium.

Figure 6.23 Preputial biopsy from a Gilbert's potoroo with balanoposthitis showing the chronic inflammatory response, secondary epithelial hyperplasia and moderate numbers of spiral bacteria (a). Haemoxylin and Eosin stain.

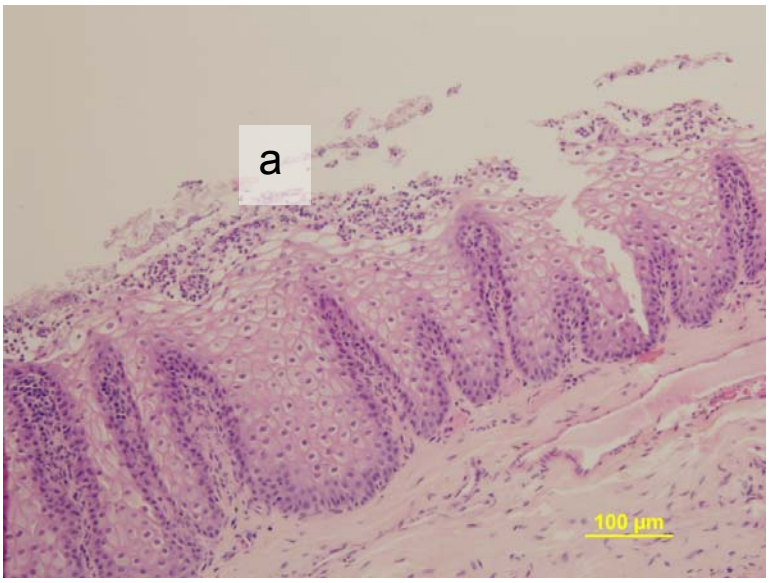
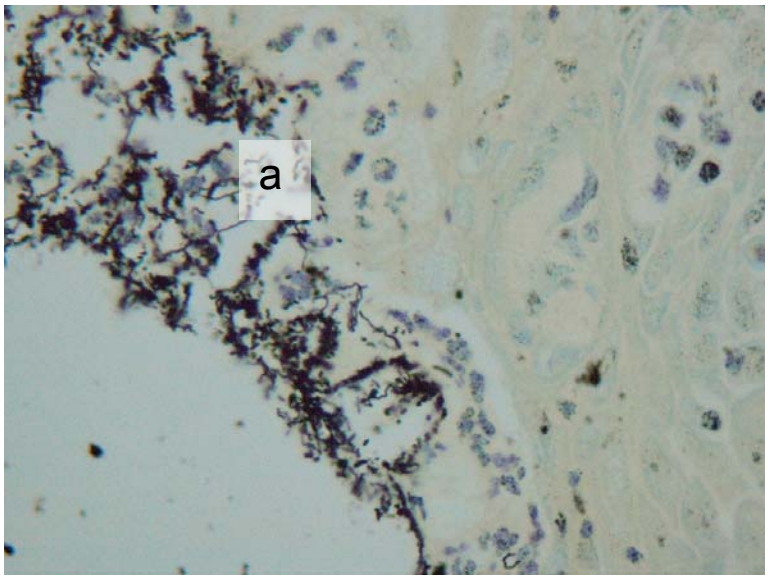


Figure 6.24 Preputial biopsy from a Gilbert's potoroo showing spirochaetes (a) diffusely scattered over and penetrating the epithelium. Warthrin-Starry stain. x300



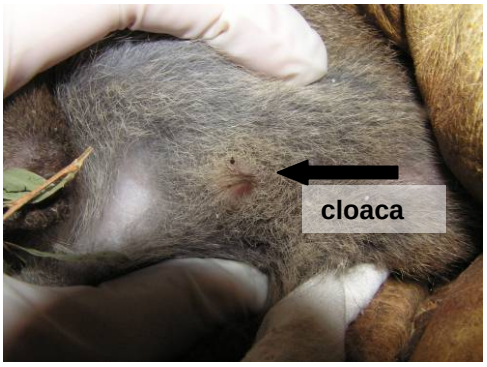
Immunohistochemistry (IHC) was conducted using commercial rabbit antibodies for *Treponema pallidum* on three occasions however the stains were unsuccessful. Control tissue for IHC was unavailable, and therefore it was not possible to determine if the IHC was unsuccessful due to the absence of *T. pallidum* or organisms with similar antigenicity to *T. pallidum* in the sections of

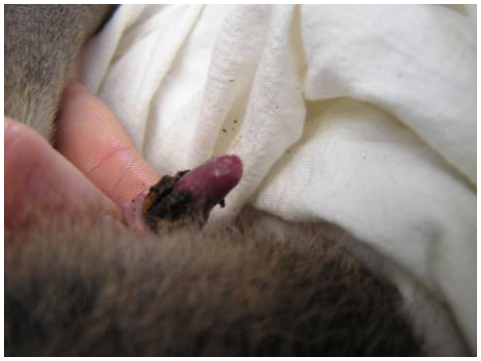
tissue from the potoroo. Poor performance of the test kit could also not be ruled out, although the internal controls for the IHC were normal.

A preputial tissue biopsy was also undertaken from a Perth zoo long-nosed potoroo at post mortem. Clinically this potoroo's prepuce had a brown adherent discharge and slight erythema (Figure 6.25). The degree of inflammation was less than that seen in the *Treponema* affected Gilbert's potoroos (Figure 6.22).

Interestingly this individual when swabbed was PCR positive for the *Treponema* in May 2006 yet in November 2006 at necropsy was negative. However, it should be noted that there was minimal material on the swab. On histopathology spirochaetes were present within the preputial tissue, however the pathologists commented that the degree of associated inflammation was minimal compared to that seen from the preputial histopathology sections in the Gilbert's potoroos.

Figure 6.25 Minimal balanoposthitis in a male captive long-nosed potoroo.



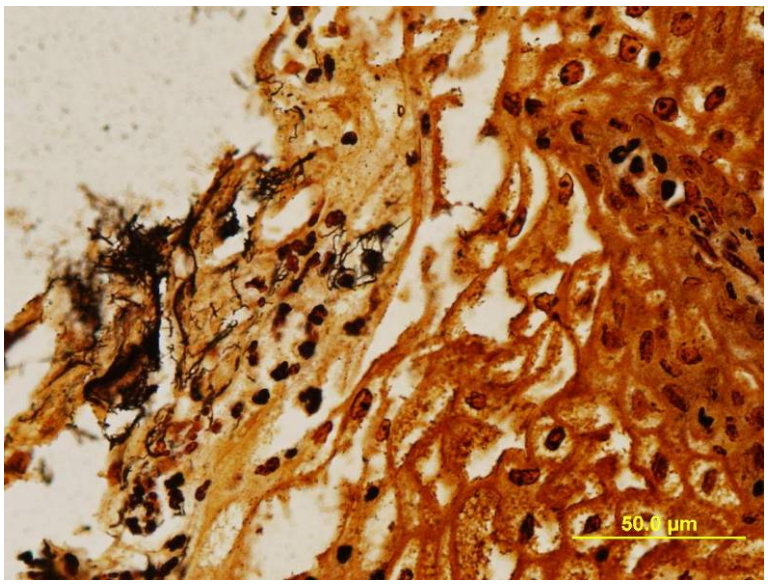


The histological lesions were described as a suppurative balanitis; chronic, focally extensive, moderate and superficial with intra-epithelial pustules. The spirochaetes were rarely associated with pustules in these sections but in places formed mats over the superficial epithelium. A moderate serocellular crust over the surfaces of the mucosa containing moderate numbers of spirochaetes and coccobacilli was found.

Penile tissue was also examined which was morphologically described to be chronic, multifocal, mild, neutrophilic and erosive with associated spirochaetes penetrating the superficial epithelium (Figure 6.26).

Small spermatoceles were present in the epididymus. These were thought to reflect sperm stasis, obstruction, epithelial degeneration and mild inflammation rather than an infectious process. However this mild inflammation was seemingly associated with the presence of the spirochaetes and so certainly may have been a factor in the formation of the spermatoceles. The predominant bacterial population in this individual from tissue biopsy culture was a moderate growth from the *Pasteurella/Actinobacillus* group and moderate growth of non-haemolytic *E. coli*. These microbiology results were very similar to that seen in the Gilbert's potoroos. No other evidence of spirochaete infection was found on necropsy.

Figure 6.26 Spirochaetes penetrating the epithelium in a penile biopsy from a long-nosed potoroo. Warthrin-Starry stain.



6.3.5 Treatment trial

Prior to commencing swabbing of the prepuce for *Treponema* PCR and microbiological culture the three male long-nosed potoroos A20406, A30237 and A40367 were weighed, condition scored and cloacal and preputial photos were taken (Figures 6.27, 6.28 and 6.29 respectively). This practice was repeated at the three subsequent treatment sessions to link clinical signs to microbiological findings.

Male A20406 presented with no inflammation of the cloaca or prepuce, and no discharge. Throughout the course of the treatment trial, there was no change, the penis and preputial tissue remained healthy.

Male A30237 presented with moderate cloacal inflammation and a sandy, moist and inflamed prepuce. A moderate amount of yellow discharge was also observed at the preputial base. In week two, the cloacal inflammation remained yet the preputial tissue was marginally less inflamed with minimal discharge at the preputial base and minor sand accumulation. This potoroo also urinated in its bag on restraint, accounting for the yellow discolouration and moistness seen surrounding the cloaca. In week three mild cloacal inflammation remained, as did mild sand accumulation and preputial inflammation. At the seven week follow up swab, the cloaca and preputial tissue appeared very similar to their initial presentation: moderate cloacal inflammation and a moist and inflamed prepuce, with a small amount of yellow preputial discharge was seen.

Male A40367 presented with mild preputial inflammation and a moderate amount of yellow-green discharge adherent to the prepuce and caudal penis. This yellow-green discharge lessened throughout the treatment trial, as did the preputial inflammation. No cloacal inflammation was evident.

Both long-nosed potoroos A20406 and A40367 cleared the *Treponema* infection following the three, weekly injections with penicillin G benzathine-penicillin G procaine and remained clear of infection one month following the trial (Table 6.30). A30237 showed moderate signs of cloacal and preputial inflammation and remained persistently infected with *Treponema* throughout the

trial. This individual was the most fractious of the three long-nosed potoroo's sampled and frequently jumped on administration of the intra-muscular penicillin injection. It is possible that this individual did not receive the full dose of penicillin in the 1st week due to this behaviour and this should be considered when assessing response to therapy.

At the three week swab at the end of the treatment trial A30237 remained persistently infected, and was the only potoroo to have moderate numbers of spirochaetes seen on dark field microscopy. At the seven week preputial swab A30237 was still reported to have moderate numbers of spirochaetes seen under dark field microscopy (Figure 6.28, Table 6.30 and Figure 6.31).

Figure 6.27 Clinical findings of male long-nosed potoroo A20406 throughout the penicillin based treatment trial for *Treponema* infection.

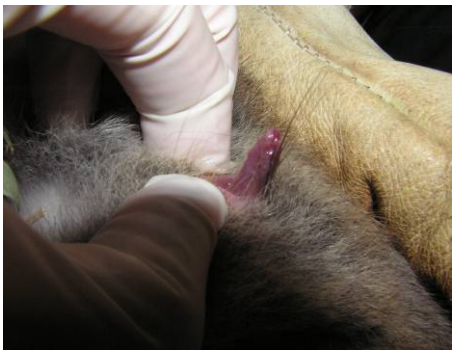
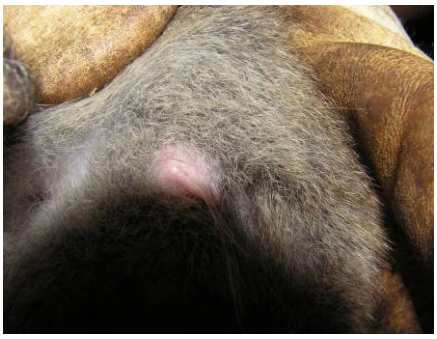
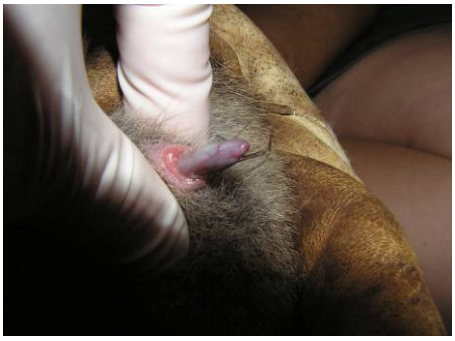
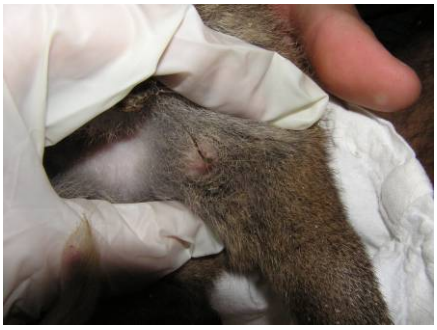
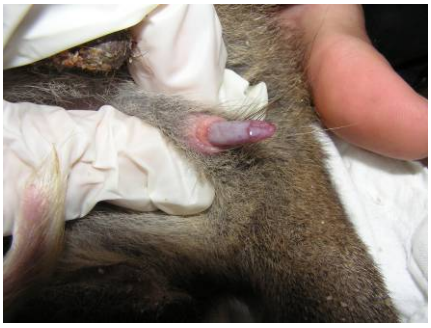
		**A20406** Week 1 Minimal cloacal or preputial inflammation No discharge Weight 1081g
		Week 2 Minimal cloacal or preputial inflammation No discharge Weight 1064g
		Week 3 Minimal cloacal or preputial inflammation No discharge Weight 1058g
		Week 7 Minimal cloacal or preputial inflammation No discharge Weight 1072g

Figure 6.28 Clinical findings of male long-nosed potoroo A30237 throughout the penicillin based treatment trial for *Treponema* infection.

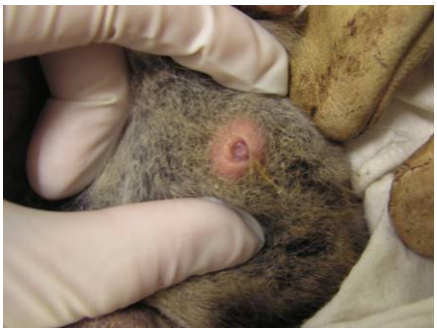
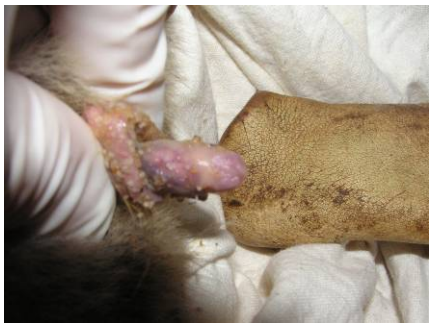
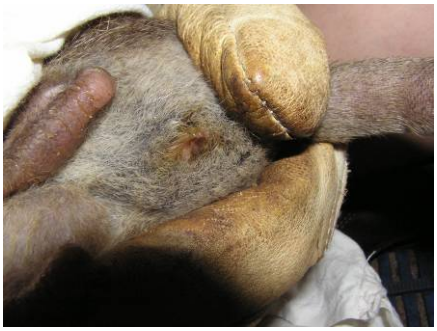
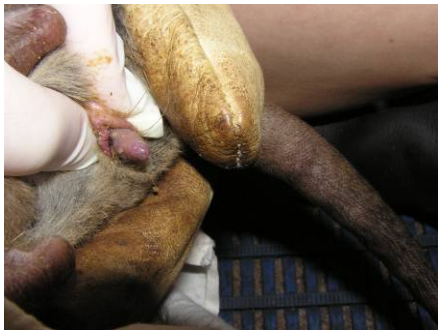
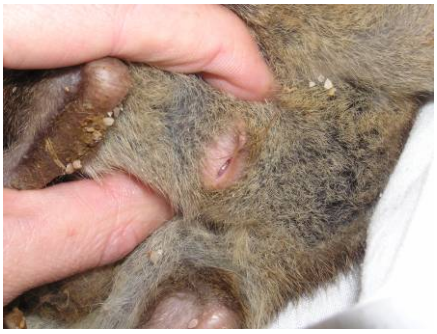
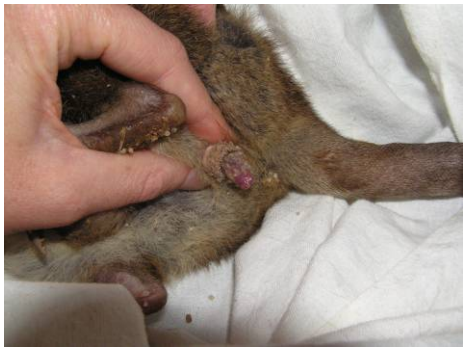
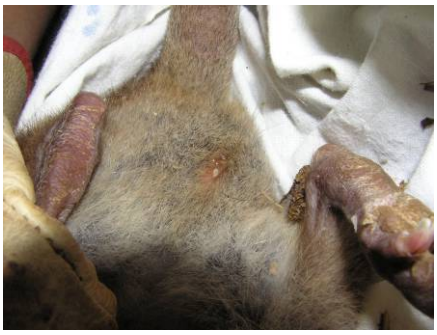
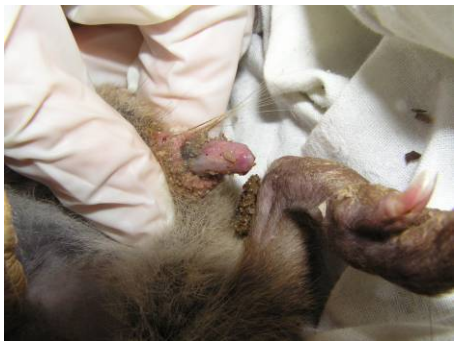
		**A30237** Week 1 Moderate cloacal and preputial inflammation, sandy prepuce, moderate yellow preputial discharge Weight 1190g
		Week 2 Moderate cloacal and preputial inflammation, sandy prepuce & moderate yellow preputial discharge Weight 1169g
		Week 3 Moderate cloacal and preputial inflammation, sandy prepuce & mild yellow preputial discharge Weight 1177g
		Week 7 Moderate cloacal and preputial inflammation, sandy prepuce & mild yellow preputial discharge Weight 1156g

Figure 6.29 Clinical findings of male long-nosed potoroo A40367 throughout the penicillin based treatment trial for *Treponema* infection.

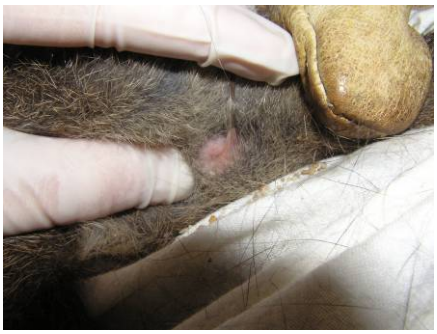
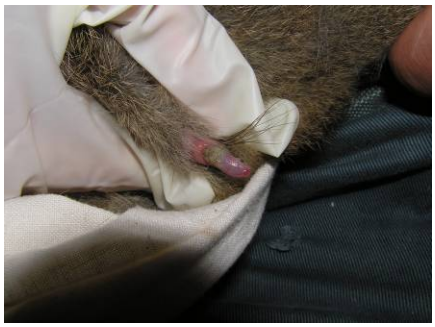
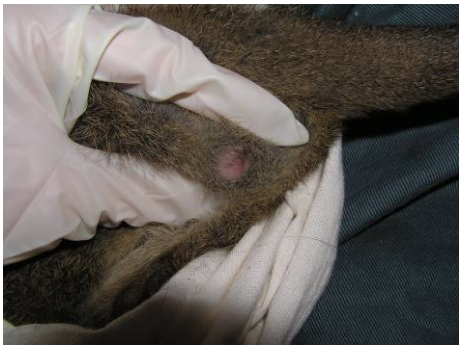
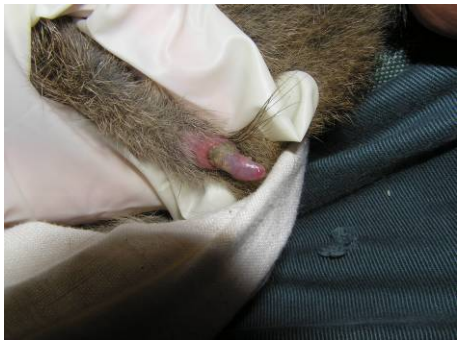
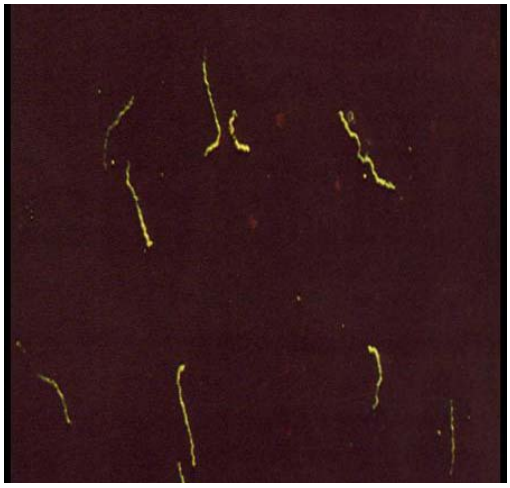
		**A40367** Week 1 Mild preputial inflammation, moderate yellow-green discharge at preputial base Weight 1039g
		Week 2 Mild preputial inflammation, mild yellow-green discharge at preputial base Weight 1012g
		Week 3 Mild preputial inflammation, mild yellow-green discharge at preputial base Weight 998g
		Week 7 Minimal cloacal or preputial inflammation, mild yellow-green discharge at preputial base Weight 1000g

Table 6.30 Preputial *Treponema* infection throughout the penicillin treatment trial in long-nosed potoroos.

Potoroo ID	*Week 1*	*Week 3*	*Week 7*	Cleared	Persistent
A20406	Pos	Neg	Neg	Cleared	
A30237	Pos	Pos	Pos		Persistent
A40367	Pos	Neg	Neg	Cleared	

Figure 6.31 Moderate numbers of spirochaetes from a preputial specimen were seen under dark field microscopy.



The corresponding microflora population was also of interest (Table 6.32). Initially all three males were PCR positive for *Treponema,* yet spirochaetes were only seen under dark field microscopy in samples from A30237 and A40367, the two long-nosed potoroos with urogenital discharge. All three had moderate numbers of *Bacteroides* sp., *Clostridium* sp., *Bifidobacterium, Pasteurella/Actinobacillus,* and *Pseudomonas aeruginosa* on preputial culture. A30237 and A40367 also had non-haemolytic *E. coli*.

Both A20406 and A40367 at the three week swab at the end of the treatment trial had a light growth of the *Actinobacillus/Pasteurella* group, whereas the persistently infected A30237 still had a moderate growth.

Table 6.32 *Treponema* infection and microbiological results throughout the penicillin treatment trial.

Species cultured in long-nosed potoroos (n=3)	Week 1	Week 3	Week 7	Number of sessions bacteria found
Aerobic				
Actinobacillus-Pasteurella group.	3	3	3	3/3 100%
Bacillus sp.			1	1/3 33.3%
Bifidobacterium sp.	3			1/3 33.3%
Enterococcus sp.		2		1/3 33.3%
Non-haemolytic E.coli	2	3	3	3/3 100%
Pseudomonas aeruginosa	3		3	2/3 66.6%
Anaerobic				
Bacteroides sp.	3		1	2/3 66.6%
Clostridium fallax	3			1/3 33.3%
Spirochaetes	2	1	1	3/3 100%
Number of species in total	**7**	**4**	**9**	**9**
Number of isolates in total	**19**	**9**	**27**	**55**

Both A20406 and A40367 also had a light growth of *Enterococcus* sp. and *Pseudomonas aeruginosa*. At this point all three had a moderate growth of non-haemolytic *E. coli.*

Results from the seven week swabs revealed heavy growths of the *Pasteurella/Actinobacillus* group in the persistently *Treponema* infected A30237 and the non- *Treponema* infected A20406. The other non- *Treponema* infected long-nosed potoroo A40367 only had a moderate growth of the *Pasteurella/Actinobacillus* group. All three had a persistent moderate non-haemolytic *E. coli* growth while the non- *Treponema* infected A20406 and A40367 had a moderate growth of *Bacteroides* sp. and *Bacillus* sp., respectively.

6.4 DISCUSSION

Epidemiological investigation of *Treponema* infection in the Gilbert's potoroo population revealed a high prevalence of infection. The period prevalence of *Treponema* infection was 72.7% (32/44).

However given two females cleared infection the true period prevalence was corrected to be 68.2% (30/44).

There was a significant difference in susceptibility to *Treponema* infection in the male compared to the female population. Males were 2.339 times more likely to have *Treponema* infection than females. This may be reflective of male anatomy whereby extrusion of the penis, as seen frequently in the captive population could abrade the mucosal surface and lead to secondary opportunistic colonization. Then when the penis is returned into its sheath and exists in a relatively anaerobic environment, anaerobic colonization occurs, creating conditions favouring the proliferation of *Treponema*. Females, on the other hand have a closed, sterile urogenital environment which is not prone to injury other than through the act of intromission. A possible mechanism for infection of females is created through intromission from an infected male or through any trauma of the cloaca causing secondary opportunistic colonization leading to conditions favouring *Treponema* colonization.

The captive population showed a generalized secular trend of increased prevalence of *Treponema* infection compared to the wild population, however this was not statistically significant. Clinically however, as described in the results section 6.3 this is of significance because if new non-infected individuals were introduced into the captive colony it is thought they would have a greater chance of developing infection, than if they were to stay in the wild. The small captive colony size (between four and eight individuals over the course of the study) and the high prevalence of *Treponema* infection in the captive population between (50-83.3%), as compared to the prevalence in the wild population (between 45.4-73.3%) illustrates this point.

A significant difference in susceptibility to *Treponema* infection in the adult compared to the sub-adult population (n = 100, Chi-Square test, p = 0.00, OR 6.044, 95%C.I. 2.434 – 15.012) was found. Adults were 6.044 times more likely to have *Treponema* infection compared to sub-adults. This may relate to transmission of infection and is supportive of venereal spread in adults through

intromission, as the sub-adults, through their very classification, are thought to be sexually immature. Correlations to human syphilis in relation to modes of transmission are of interest in relation to these findings. *T. pallidum* rapidly penetrates intact mucous membranes or microscopic lesions in the skin and within hours enters the lymphatic system and bloodstream to produce systemic infection long before the appearance of a primary lesion. The site of entry may be vaginal, rectal or oral. *T. pallidum* is not very viable outside its host, so syphilis is ordinarily acquired by sexual intercourse. Less common modes of transmission include nonsexual personal contact, *in utero* infection, and blood transfusion. Incubation time is inversely proportional to the number of organisms. However, the period of time from the point of inoculation to the development of a primary lesion is usually about 4–6 weeks. In comparison *Treponema paraluis-cuniculi* in rabbits has been found to be spread via vertical, direct and venereal contact, and carriers may be asymptomatic until stress occurs (DiGiacomo *et al*. 1983, Saito *et al.* 2003). Incubation time is dependent on age but in adults following intradermal inoculation, lesions developed three to five weeks later (Cunliffe-Beamer and Fox 1981). Interestingly neonatal rabbits have been reported to respond variably to adults when experimentally inoculated. Lesions have not been reported in naturally infected rabbits until 10 weeks of age. At this time endocrine changes allowing response to exogenous stimuli became active (Fox *et al*. 1964). It was theorised that neonatally inoculated rabbits became tolerantly infected, yet the lack of immune stimulation meant that lesions did not develop (Cunliffe-Beamer and Fox 1981). Protection of the neonates by maternal antibody was ruled out as the dams were clinically and serologically free of the disease. Although no Gilbert's potoroos under 400g were sampled in this study (under 125 days or 18 weeks), a higher prevalence of infection was certainly evident in the adult compared to the sub-adult population. It remains unknown as to whether immune tolerance plays a role in altering response to disease in sub-adult potoroos. The age at which a Gilbert's potoroo acquires a functional immune system is yet to be determined, however the interesting case-study of captive female (F93) outlined below, provides further insight into modes of transmission of *Treponema* in the Gilbert's potoroo.

Female GP 93 a hand-reared potoroo, was brought into the captive colony as a sub-adult in March 2005. This individual showed no presence of discharge and was PCR negative for the *Treponema* infection, until June 2006 when she became PCR positive for *Treponema* infection and developed a 1+ discharge. GP F93 would have become reproductively active and classified as an adult around January 2006. In February 2006 she was paired with captive M46, a male which was persistently PCR positive for *Treponema* infection since March 2005 and showed variable 2+ to 3+ discharge over this time. At the following sampling session (June 2006) GP F93 became PCR positive for *Treponema* infection. It seems most likely modes of transmission would include venereal, or direct non-sexual contact. The fact that these individuals were housed together may increase the potential for direct contact and infection transfer, however *Treponemes* are typically not viable outside their host, and have very specific growth requirements, which makes direct contact a less likely option. Furthermore, the urogenital orifice of the female Gilbert's potoroo is small, and is unlikely to be penetrated other than through the act of intromission. Additionally no blood transfusions have been undertaken in Gilbert's potoroos. Therefore sexual intercourse seems the most likely scenario for urogenital transmission of *Treponema* infection between adults.

Although *Treponema* infection in sub-adults is far lower in prevalence than in adults. It is still present. This suggests that modes of transmission similar to those reported in rabbits may be operating, such as vertical transmission, or direct contact post-partum. Alternatively the individual could have reached sexual maturity at an earlier stage than currently anticipated.

In March 2007 a Gilbert's potoroo pouch young (whose mother was PCR positive for the *Treponema* infection was cross-fostered into a long-nosed potoroo (who was PCR negative for the *Treponema* infection) and the pouch young subsequently developed a green discharge and was PCR positive for *Treponema* infection. This finding is highly interesting given the timing of the cross-fostering and the potential stressors associated with cross-fostering. Possible theories for the infection in the pouch young could include (a) vertical infection which has become clinical

following cross-fostering, possibly associated with immune function (b) direct contact from the infected mother, again which has become clinical following cross-fostering (c) cross-infection from the surrogate mother long-nosed potoroo, (unlikely given she was PCR negative and had no discharge) (d) sexual transmission at this young stage (this was not a possible option, as the pouch young had not been exposed to a male potoroo).

It would be an interesting exercise to conduct further studies on modes of transmission. However, due to the lack of genetic variation amongst Gilbert's potoroos it is not feasible to conduct genetic analysis of wild individuals to determine kinship relationships. Furthermore, captive individuals have not successfully mated since 2002 and so infection transfer to offspring and sub-adult infection cannot be further studied at this point.

However, the reproductive consequences of *Treponema* infection and potential impact on population dynamics have been studied. Females with evidence of *Treponema* infection appeared to still be reproducing in November 2005. At this time four adult females with pouch young showed no evidence of urogenital discharge, yet had low numbers of spirochaetes seen under dark field microscopy and were PCR positive for *Treponema* infection. Only F100, a young adult female, in March 2007 remained PCR positive and lacked pouch young. This individual, also had evidence of 1+ green urogenital discharge, and low numbers of spirochaetes on dark field microscopy. At this time the other two females (GP F69 and GP F89) were re-trapped, and had no evidence of discharge or evidence of spirochaetes on dark field microscopy; both of these females had pouch young.

It is possible that GP F69 and GP F98's immune systems were able to mount an effective immune response to clear the infection, while GP F100 for some reason was unable to clear the infection, developed urogenital discharge and became persistently infected. It is also interesting that GP F100's first breeding season would have been November 2005, where as the other three females were seasoned breeders. In November 2005, F100 had a slight leukopaenia and

evidence of the haemoparasite *Theileria* on haemoparasite examination yet no other evidence of disease was found on physical exam and subsequent serological screening studies were all negative. Although based on a very small sample size, the results from these females may suggest that age of infection, or number of breeding seasons has an association with females either clearing *Treponema* infection or remaining persistently infected. A longer sampling period is required to investigate this theory further.

Over the course of the study 44 Gilbert's potoroos were sampled. Two adult females (one wild and one captive) cleared the infection. No males cleared infection. Seven males and one female, once infected, became persistently infected. Two individuals were swabbed throughout the trial and had an overall negative (-) status (wild adults GP F86 and GP M98). Female GP 86 was not recorded with pouch young throughout the study and the possibility of other factors (anatomic, disease or social) limiting reproductive function should be considered, although no abnormalities were detected on health screening of this individual. No clinical signs of inflammation, urogenital discharge or evidence of *Treponema* infection was found using dark field microscopy for spirochaetes or through urogenital swabs for *Treponema* PCR in five southern brown bandicoots, three yellow footed antechinus and two quokkas. Disease transfer among Australian wildlife species, in particular marsupials is a growing area of interest. Zoonotic infections where wildlife species act as reservoirs of infection are of particular importance. However, the lack of infection in con-specifics points to a potoroo-specific *Treponema* infection.

A significant relationship between the presence of discharge and *Treponema* infection was found (n = 100, Chi-Square test, p = 0.00, OR 26.87 95%C.I. 8.64-83.51). A significant relationship between PCR results and *Treponema* infection was found (n = 100, Chi-Square test, p = 0.00, OR 4.54, C.I. 2.37 – 8.67). Likewise a significant relationship between the level of spirochaetes and *Treponema* infection, was found (n = 100, Chi-Square test, p = 0.00). All diagnostic tests undertaken for *Treponema* infection therefore, had a statistically significant association with the disease. However there were a relatively high number of false PCR negative results. Reasons

proposed for this result include delayed transport and associated heating of samples in transit and the possibility of inhibitors in the smegma or vaginal secretions. This second factor requires further investigation to optimize this assay

A generalized trend of higher percentage rates of moderate (2+) and severe (3+) clinical signs and levels of discharge in males with *Treponema* infection was observed, while *Treponema* infected females either showed no discharge or had 1+ discharge over the course of the study. Correspondingly, a significant relationship between the level of discharge and gender, was found (n = 100, Chi-square test, p = 0.00, O.R 26.87 95% C.I 9.18-78.26). This may be related to the anatomic differences in the male and female urogenital tract. Females are less subjected to ongoing trauma to their urogenital tract and so have less likelihood of subsequent bacterial colonization from disturbances in mucosal integrity. The male's ability to evert, retract and possibly abrade mucosal barriers creates an obvious avenue for mucosal colonization and commensal overgrowth, which in turn promotes a favourable micro-environment for *Treponema* growth. This hypothesis is strengthened by the fact that males compared to females, as discussed in 5.3.2.3 had a greater or equal number of anaerobic bacterial isolates and species than females, in all trapping sessions. Alternatively, specific bacteria could be involved. *Actinobacillus* and *Pasteurella* had a statistically significant relationship to *Treponema* infection and their higher prevalence in males compared to females discussed in 5.3.2.3 and 5.3.2.5 may be a factor leading to the presence of higher magnitudes of discharge in *Treponema* infected males.

Interestingly, there was no statistical association between the presence of clinical signs and discharge with origin (wild or captive individuals) (n = 100, Chi-Square test, p = 0.226). This is surprising as when the three most prevalent aerobes isolated from the urogenital tract were compared (5.3.2.9) these species were found in a greater number of trapping sessions in the wild compared to the captive population. *Actinobacillus* sp. was found in the wild population in 5/6 trapping sessions (83.3%), compared to the captive population where *Actinobacillus* sp. was

found in 4/6 trapping sessions (66.7%). *Corynebacterium pilosum,* like *Pasteurella,* was more prevalent in the wild population in all trapping sessions. In the captive colony it was present in 3/6 trapping sessions (50%). *Pasteurella* was present in 5/6 trapping sessions (83.3%). The reason for this finding is unknown, however statistically speaking it seems the origin of the Gilbert's potoroo (wild vs captive) is of no significance in relation to the clinical signs and level of discharge observed.

Overall the presence of discharge was associated with a polymicrobial environment containing *Treponema*, *Actinobacillus* and *Pasteurella*. *Treponema* infection was most positively associated with the presence of discharge, however those lacking *Treponema* infection with *Actinobacillus* infection (OR 25.69 95% C.I 7.33-90.05) and/or *Pasteurella* infection (OR 19.73 95% C.I 6.17-63.08) still presented with discharge. This suggests that like human periodontitis environmental enrichment for *Treponema* by preceeding bacterial populations and subsequent inflammatory events seems crucial to the underlying pathology, and also suggests that the presence of discharge while often a precursor of infection is not a method of definitive diagnosis.

Treponema infection was defined by clinical signs and the presence and nature of the discharge, PCR positivity and dark field microscopy for spirochaetes. When these three techniques were analysed for their sensitivity and specificity, dark field microscopy for spirochaetes was the most sensitive test (100%) and the most specific test conducted was PCR (100%) (Appendix 12).

Therefore, there is not one gold standard test, rather it is recommended that all three tests are undertaken, and the results then tabulated to help diagnose *Treponema* infection. For example, a male potoroo that has severe clinical signs and discharge (3+), and evidence of spirochaetes on dark field microscopy, but that is PCR negative is likely to have *Treponema* infection. The PCR result should be classed as a false negative and other PCR results taken on that day should also be interpreted with caution.

Of all the three tests, clinical signs and the presence of discharge is probably the most simple to comment on with reference to the balanoposthitis chart (Appendix 4). However, the low specificity of this test necessitates cross-referencing with dark field microscopy and PCR to diagnose infection. Dark field microscopy is a relatively simple technique to conduct if a dark field microscope is available in a field station, however due to the fastidious nature of *Treponema* it is recommended that slides are analysed within 24 hours of collection. This technique also relies on operator experience in detecting the spiral shaped organisms. PCR is the most costly of all techniques and requires considerable experience or a dedicated laboratory to process samples. In the field situation, it is easiest to collect swabs and immediately place them on ice, store the swabs frozen, and then batch and send to the laboratory at the end of the trapping session. However, it is imperative that these samples are kept at -20º C until reaching the laboratory, as the freeze-thaw cycle can result in a loss of biological activity (N. Buller personal communication, December, 2007). Three PCR samples collected during the March 2007 trapping session were most likely affected in this way due to the prolonged courier service on this occasion, and therefore these samples were classed as false negatives.

The combined prevalence for the autumn, winter and spring trapping session was 57.1% (20/35), 70% (21/30) and 65.7% (23/35), respectively. The highest prevalence of infection of 70% (21/30) was seen over the June (winter) trapping sessions 70% (21/30). This may reflect potoroo burrowing behaviour, where dirt and detritus hides are created for protection against inclement weather, providing a warm and moist environment. These environmental factors may also favour growth of secondary microbes to form optimal conditions for *Treponema* growth. Further validation and sampling is required to confirm this. However, a one-way between groups ANOVA with planned comparisons to explore the prevalence of *Treponema* infection over the June (winter) trapping sessions compared to the March (autumn) and November (spring) trapping sessions reported no statistically significant difference in *Treponema* infection over the June (winter) season, compared to March (autumn) or November (spring) was found ($F(1, 53.502) = 0.714$, $p = 0.402$).

6.4.1 PCR and sequencing

When product sequenced from the SPF5 and SPR4 primers was identified using BlastN and Genbank a small sequence of approximately 190 base pairs revealed a 92% maximal identity to an uncultured *Treponema* clone. The 16S rRNA gene was used to identify the bacteria for several reasons. Firstly taxonomists today consider analysis of an organism's DNA more reliable than classification based solely on phenotypes. Secondly, researchers may, for a number of reasons, want to identify or classify only the bacteria within a given environmental or medical sample. While there is a homologous gene in eukaryotes (the 18S rRNA gene), it is distinct, thereby rendering the 16S rRNA gene a useful tool for extracting and identifying bacteria as separate from plant, animal, fungal, and protist DNA within the same sample. Thirdly, the 16S rRNA gene is relatively short at 1.5 kb, making it faster and cheaper to sequence than many other unique bacterial genes (Barton 2005).

However, as Paster *et al*. (1991) suggested, phylogenetic clustering of spirochaetal groups rather than specific percent similarity was then used to define spirochaete genera based on the results of original sequencing back in 2002, and the sequencing conducted in this study.

The findings of this phylogenetic study identified the organisms identified in 2002 to be the same. Product from the original sequencing conducted in 2002 was most closely associated with the non-cultivated *Treponema medium* subsp. *bovis*, *Treponema medium* genomic RNA for 16S ribosomal RNA and *Treponema* sp. oral clone JUO31.

In periodontal disease in humans and DD, cultivated species and uncultivated phylotypes have been detected. In DD lesions in sheep and cattle these polymicrobial infections have been grouped into five phylogenetic clusters (Choi *et al.* 1997). The only cultivable species in DD lesions is *Treponema brennaborense,* however other cultivable *Treponema* are closely related to the human-oral associated *T. denticola*, *T. medium/vincentii* and *T. phagedenis* (Stamm and Trott 2006). *T. medium/vincentii* has been shown to be invasive and tissue-destructive in an *in vivo*

animal model (Kesavalu *et al.* 1997). Based on partial 16S sequencing we have identified a Treponeme with 92% maximal sequence homology to an uncultured *Treponema* clone. Further analysis on Genbank revealed this organism to have the most similarities to environmental samples, 10 hits from 5 organisms: *Treponema phagedensis*, 15 hits from 2 organisms and *Treponema phagedenis* subsp. *vaccae* 14 hits from 1 organism (equivalent name *Treponema bovis*).

Therefore it appears from partial 16S sequencing that the *Treponema* infection found in the Gilbert's potoroo is most similar to the *Treponema* phylotypes associated with papillomatous digital dermatitis found in cattle and sheep. The two groups most similar to the sequence results found in Gilbert's potoroo are group one, which appear to closely cluster with oral species *T. medium/vincentii* and group 3 which is identical to *T. phagedenis* (Figure 6.21).

Cross-referencing of results of SPF5 and SPR4 primers with TTF and TTR primers found differences suggesting that multiple Treponemes may be involved in the pathogenesis of balanoposthitis in the Gilbert's potoroo. When the PCR results of the TT primers and SPF5 and SPR4 were compared, five individuals that were TT positive were SPF5 and SPR4 negative. However, the results from sequencing the TT forward and reverse primers had a 98% maximum identity with an uncultured Porphytomonadacea clone. A genus within this family is *Porphyromonas*. It was assumed that the TT primers cross-reacted with *Porphyromonas* sp. and so it is recommended that SPF5 and SPR4 primers are used for molecular testing of future potoroo samples

Porphyromonas sp. inhabits the oropharynx, nose, gastrointestinal and urogenital tract in humans. However, *Porphyromonas* sp. has also been isolated from a brain abscess, liver abscess (Jover-Diaz 2003), sinusitis, osteomyelitis (Duerden 1993), dental-oral bacteraemia, inflammatory pelvic disease, balanitis, endometritis and cutaneous and soft tissue infections (Melon *et al.* 1997)(Chapter 5.1.3.14). However the role that *Porphyromonas* sp. plays in the

observed balanoposthitis in Gilbert's potoroo is thought to be similar to the role *Porphyromonas* sp. potentially plays in bovine digital dermatitis and human periodontal disease. In bovine digital dermatitis, biopsies from hoof lesions were cultured and a multitude of Gram-negative bacilli were found including *Fusobacterium necrophorum*, *Fusobacterium nucleatum*, *Porphyromonas levii* and a variety of *Prevotella* sp. (Koniarova *et al*. 1993). Likewise in periodontal lesions, *T. denticola* is typically found in association with obligate anaerobic bacteria including *Porphyromonas gingivalis* and *Bacteroides forsythus* (Socransky *et al.* 1998). These anaerobes are thought to interact with surrounding periodontal tissues to facilitate adhesion to epithelial cells and extracellular matrix components, contribute to proteolysis and modulate host immune function to produce pathology (Lamont and Jenkinson 1998). It is therefore hypothesized that *Porphyromonas* sp. in the Gilbert's potoroo, like *Porphyromonas* sp. involved in bovine DD and human periodontal disease, provides environmental enrichment for *Treponema* in the Gilbert's potoroo through preceeding bacterial anaerobic population and subsequent inflammatory events.

Further studies are needed to define the contribution of the Gilbert's potoroo *Treponema* phylotype (alone or in combination) to the initiation and progression of balanoposthitis. These studies are currently hampered by the inability to conduct inoculation studies given the critically endangered nature of the Gilberts potoroo and the inability to cultivate the *Treponema* present in balanoposthitis lesions.

6.4.2 Serology

No antibody response was detected to any of the serological tests. There are many reasons why this may have occurred.

The RPR is a heterophile test and the relevant antigen and antibody responses may not occur in non-human species. The complexities of the marsupial immune response should also be taken into account especially given that these responses are still being further characterized. Young

(2003) performed immune function testing on tissues recovered from the analogous long-nosed potoroos and stated that the immune response in this species seemed to mirror the response seen in eutherians. Mucosal host resistance may also be involved in the lack of serological response. Very little work on mucosal immunity in marsupials has been conducted, other than Doolin *et al.* (2002) who showed that following intranasal immunization, antigen-specific antibodies can be detected in secretions from the female reproductive tract of brushtail possums. However the persistence of pathogens in the reproductive tract without systemic disease is a feature of other venereal diseases such as Campylobacter and Triitrichomonas, and therefore the failure of the human Treponema serologic tests may reflect that antibodies are present in local secretions but not in serum.

Furthermore in humans, non-treponemal tests lack sensitivity in late stage infection. 30% of patients with late latent or late active syphilis will show a non-reactive result (Nesteroff 2004).

Additionally 1-2% of patients with secondary syphilis exhibit a prozone reaction (Nesteroff 2004). This occurs when an excess of antibody in undiluted serum inhibits flocculation with the antigen. This results in weakly reactive, atypical or occasionally false negative results.

Ideally testing should be repeated to determine if any of the above factors, including late latent or late active syphilis or a prozone reaction resulting in false negative results is occurring. An alternative hypothesis is that *Treponema* in Gilbert's potoroos is only locally invasive in the urogenital tract with no systemic infection occurring.

6.4.3 Histopathology and immunohistochemistry

The severe balanoposthitis in two *Treponema* infected captive individuals with histopathology lesions consisting of a moderate, chronic inflammatory response with secondary epithelial hyperplasia in conjunction with mild microabsessation, penile erosion, and moderate numbers of spirochaetes suggested a strong causative relationship between the presence of spirochaetes and balanoposthitis. These spirochaetes were approximately 2-4µm in length, silver stained and

were associated with a moderate accumulation of inflammatory cells, predominantly macrophages, with lesser numbers of lymphocytes and plasma cells. Associated culture revealed a heavy mixed growth of the *Actinobacillus-Pasteurella* group and non-haemolytic *E. coli,* all commonly found isolates in preputial culture of *Treponema* infected Gilbert's potoroos. Whilst non-haemolytic *E. coli* is thought to be a commensal, secondary opportunistic organism, *Actinobacillus* and *Pasteurella* are thought to either play a role in the polymicrobial relationship optimizing conditions for the growth of *Treponema* or alternatively, *Treponema* infected individuals have factors causing reduced host compromise which promote *Actinobacillus* and *Pasteurella* infection. The former seems more likely as the penile erosions were associated with a mixed bacterial population, however the presence of intra-epithelial spirochaetes strongly suggested the spirochaetes were the primary infection (S.Besier, personal communication, January 2007).

Clinically the Perth zoo long-nosed potoroos had no evidence of green preputial discharge, instead it was brown with only minimal surrounding penile erythema. Overall the degree of inflammation was far less than that seen in the *Treponema* infected Gilbert's potoroos and there was no evidence of associated ulceration or microabscessation.

The histological lesions in the long-nosed potoroo that was necropsied were also milder than those seen in the Gilbert's potoroos. A suppurative balanitis; chronic, focally extensive, moderate and superficial with intra-epithelial pustules, was described.

The predominant bacterial population in this long-nosed potoroo from tissue biopsy was a moderate growth from the *Pasteurella/Actinobacillus* group and moderate growth of non haemolytic *E. coli*, very similar to that seen in the Gilbert's potoroos.

Therefore, it seems a similar microbiological environment is required for colonization with *Treponema* in both species, with the presence of intra-epithelial spirochaetes in the Gilbert's potoroos suggesting the spirochaetes were the primary infection. The associated tissue reaction in the Gilbert's potoroo compared to the long-nosed potoroo was far greater, possibly reflective of

a more pathogenic species of *Treponema* in the Gilbert's potoroo. Alternatively immunosuppression in the Gilbert's potoroo could explain the greater clinical consequence and tissue reaction. However, the presence of inflammatory cells evident surrounding the spirochaetes in the Gilbert's potoroos would indicate an immune response was occurring, it may, however, not have been adequate.

The similarity in histopathology findings of *Treponema paraluis-cuniculi* to *Treponema* infection in the Gilbert's potoroo is striking. Ulceration and hyperkeratosis of the epidermis with variable inflammatory components is observed in both species. Epidermal microabsesses or vesicles were occasionally observed. The predominant dermal lesion was infiltration of plasma cells or macrophages (Cunliffe-Beamer and Fox 1981). Diagnosis in rabbits depends upon finding *Treponema paraluis-cuniculi* in suspect lesions or demonstrating positive serological tests. The lack of antibody response in serological tests of samples from Gilbert's potoroos should not rule out the diagnosis of infection, as the characterisation of the marsupial immune response is still under investigation. In rabbits, a definitive diagnosis is substantiated through observing spirochaetes under dark-field microscopy or via histopathological exam, although false negatives can occur with dark field microscopy if only a few spirochaetes are present. Therefore, a diagnosis of *Treponema* infection in the Gilbert's potoroo should be made if *Treponema* can be visualized through dark field microscopy and silver stained spirochaetes are visible with the described histopathological changes and a positive PCR result is obtained.

Due to the lack of control tissue for IHC it was not possible to determine if the IHC was unsuccessful due to the absence of *T. pallidum* or organisms with similar antigenicity to *T. pallidum* in the sections of tissue from the potoroo. Poor performance of the test kit could also not be ruled out, although the internal controls for the IHC were normal. Given the samples tested had evidence of spirochaetes on histopathology, the lack of response may be linked to the above factors or the lack of specificity of the Gilbert's potoroo sample to the rabbit commercial *T. pallidum* antibody. Veterinary pathologists often face many challenges in conducting IHC as

there are no guarantees that antibodies will cross react amongst various species (Ramos-Vara 2005).

6.4.4 Treatment trial

All three members of the long-nosed potoroo colony at the Perth Zoo initially had PCR evidence of *Treponema* infection. Long-nosed potoroo A20406 showed no associated clinical signs. Long-nosed potoroo A40367 had mild preputial inflammation which lessened back to normal over the trial. This individual also had a small amount of yellow-green discharge (1+), which lessened throughout the trial. The long-nosed potoroo A30237 had moderate cloacal inflammation, a moderate amount of yellow preputial discharge and moderate preputial inflammation. The amount of discharge and cloacal and preputial inflammation lessened throughout the trial, however this individual remained PCR positive despite penicillin therapy. The moderate associated balanoposthitis in this individual was also indicative of a more severe inflammatory response, therefore a higher dose rate of penicillin may have been required to clear infection. In humans with chronic infections three weekly injections of 2.4 million IU of penicillin G benzathine are administered. While in rabbits with chronic *Treponema paraluis-cuniculi* three weekly doses of 66 700 IU (with a total dose of 200 000 IU/kg) were required to clear infection. Long-nosed potoroo A30237 received 84 000 IU/kg for three weeks (a total dose of 252 000 IU/kg). This should have been well in excess of needs when extrapolated from the rabbit model. However, the possibility that this fractious potoroo did not receive the full dose of the initial injection should be considered as a potential reason for treatment failure. Alternatively, chronically infected potoroos may require a higher dose rate than rabbits, a definite possibility given the complexities in characterizing the marsupial immune response. The other two males A20406 and A40367, cleared the *Treponema* infection and remained clear one month post-trial completion. A20406 appeared clinically normal throughout the trial and A40367 had mild inflammation of the prepuce and a mild yellow-green preputial base discharge which improved over the course of treatment.

The associated microflora burden was similar to that reported in Gilbert's potoroos. In Gilbert's potoroos the *Treponema* infected population was 3.343 times more likely to have a *Pasteurella* infection than the non-*Treponema* infected population, and was 5.824 times more likely to have *Actinobacillus* than the non-*Treponema* infected population. All long-nosed potoroos throughout the trial had evidence of microbial growth from the *Pasteurella* and *Actinobacillus* group, however the *Treponema* infected individuals had a higher magnitude of growth than the non-*Treponema* infected individuals. Like the Gilbert's potoroo this may reflect that *Actinobacillus* and *Pasteurella* play a role in the polymicrobial relationship optimizing conditions for the growth of *Treponema* or that factors causing reduced host compromise are present in *Treponema* infected individuals promoting *Actinobacillus* and *Pasteurella* infection. However, the continued presence of *Actinobacillus* and *Pasteurella* in long-nosed potoroos which had cleared infection makes it more likely that these two bacteria are playing a role in the polymicrobial relationship optimizing conditions for the growth of *Treponema*. The increased magnitude of *Actinobacillus* and *Pasteurella* in the persistently *Treponema* infected long-nosed potoroo compared to those that had cleared the *Treponema* infection adds weight to this statement. However, relating back to the histopathology findings in the necropsied long-nosed potoroo, penile pustules associated with a mixed bacterial population including *Actinobacillus*, *Pasteurella* and non-haemolytic *E. coli* with the presence of intra-epithelial spirochaetes were found suggesting that the spirochaetes were the primary infection.

The presence of *Pseudomonas aeruginosa* is of interest as at week three it was only present in the two non-*Treponema* infected males A20406 and A40367. However, at the seven week swab all three males were infected. This may reflect contamination of the urogenital tract with *Pseudomonas* due to reduced numbers of normal flora associated with the penicillin treatment. Likewise the moderate growth of non-haemolytic *E. coli* in infected and non-infected individuals was thought most likely to be representative of opportunistic colonization as a small growth of non-haemolytic *E. coli* is thought to be commensal in most species.

The anaerobic population in the long-nosed potoroos did not seem to be as prolific as that seen in the Gilbert's potoroos with *Treponema* infection. However initial week one culture results revealed all long-nosed potoroos to have significant anaerobic cultures from which *Bacteroides* sp. and *Clostridium* sp. were isolated in conjunction with PCR positive *Treponema* findings. No anaerobes other than *Treponema* were found in weeks three and seven in A30237, although A20406 had a moderate growth of *Bacteroides* sp.

It seems in potoroos with mild clinical signs of *Treponema* infection penicillin G benzathine-penicillin G procaine at 84 000 IU/kg IM at seven day intervals for three injections is effective. However, in those with moderate infections a higher dose may be required.

In relation to treatment in the Gilbert's potoroos it is recommended that pencillin G benzathine is administered as a trial to the remaining captive colony at 100 000 IU/kg weekly for three weeks given they are chronically infected with *Treponema*. If this is successful then it is recommended that all Gilbert's potoroos that undergo translocation be quarantined in separate pens in the captive colony for three weeks prior to translocation to receive pencillin G benzathine according to the regime described above to enable the establishment of a non-*Treponema* infected breeding colony. This will not only ensure disease is contained, it will also allow direct comparisons in reproductive rate in an infected and non-infected colony. At this point in time it does not seem feasible to attempt to eradicate disease in the wild population, as this would require that all wild Gilbert's potoroos be trapped weekly for three weeks. It would be difficult to ensure this task was achieved as although the potoroos do not appear trap shy, all individuals from the entire population are never trapped during one trapping session. From a conservation perspective, it seems highly important to eradicate the disease from the enzootically infected captive colony. Furthermore, when we consider the condition in the Gilbert's potoroos in light of the rabbit model two important facts are highlighted; the associated genital lesions considerably reduced reproductive capabilities through dyspareunia, and *Treponema paraluis-cuniculi* was thought to alter immune response as evidenced by the development of severe *Pasteurella* infection soon after severe clinical vent disease became apparent (Cunliffe-Beamer and Fox

1981). These findings reiterate that the disease in Gilbert's potoroos should not be ignored. Especially when similar alterations in immune responses have been reported in rabbits experimentally infected with the closely related *Treponema pallidum* (Shell and Musher 1974).

Throughout this study on health and disease, numerous diseases have been identified as having clinical significance with regards to population dynamics. If the presence of *Treponema* is altering immune responses, it is likely that if it remains untreated previously mentioned diseases, or new diseases, may exert greater clinical effects and have greater consequences for the Gilbert's potoroo meta-population. Therefore, treatment of *Treponema* infection, undertaken in accordance with the recommended treatment regime outlined above, seems of utmost importance for the survival of this critically endangered species.

6.5 CONCLUSION

A novel *Treponema* sp. has been isolated, sequenced and defined in the Gilbert's potoroo. The period prevalence of infection was 68.2% (30/44) over the three year study period. In males spirochaetes were only present in individuals with discharge and were consistently associated with balanoposthitis. Males had a statistically significant higher prevalence of infection compared to females, and adults also had a statistically significant higher prevalence of infection compared to sub-adults. Human and animal models of infection were studied, and venereal or horizontal contact in adults and vertical or horizontal transmission in sub-adults, were considered to be the most likely means of transmission.

It is difficult to comment on the reproductive effects of *Treponema* infection in the Gilbert's potoroo, as both the wild and captive population are infected and there is no control group for comparison. However four infected adult females with *Treponema* infection are still reproducing, and one infected young adult is not. While the disease does not cause severe debilitation, in males the presence of *Treponema* infection can be associated with a severe balanoposthitis,

often making extrusion of the penis physically difficult. The clinical signs are strikingly similar to that seen in rabbits, where genital lesions contributed to reduced reproductive capabilities through dyspareunia (Cunliffe-Beamer 1981). Colony outbreaks in rabbits reportedly result in a decreased rate of conception and increased rate of metritis, placenta retention and neonatal deaths. Furthermore, the possibility of alterations in immune function increasing susceptibility to secondary disease should not be overlooked.

This novel *Treponema* is best screened for by PCR, in conjunction with dark field microscopy for spirochaetes. The level of discharge should also be taken into account. Histopathology and silver staining provides definitive diagnosis. The *Treponeme* was not able to be cultured and serological screening through the human RPR, TPPA and the *T. pallidum* total Ab test was non-diagnostic. Additionally, immunohistochemistry was inconclusive.

Overall, the presence of discharge was associated with a polymicrobial environment containing *Treponema*, *Actinobacillus* and *Pasteurella*. Presence of discharge was most positively associated with *Treponema* infection, although those lacking *Treponema* infection with *Actinobacillus* infection and/or *Pasteurella* infection sometimes presented with discharge. The continued presence of *Actinobacillus* and *Pasteurella* in long-nosed potoroos, which had cleared *Treponema* infection reflect that these two bacteria are playing a role in a polymicrobial relationship to optimize conditions for the growth of *Treponema*. The increased magnitude of *Actinobacillus* and *Pasteurella* in the persistently *Treponema* infected long-nosed potoroo, compared to those that had cleared the infection also added weight to this statement.

Captive potoroos, and potoroos to be translocated with mild clinical signs of *Treponema* infection should be treated with penicillin G benzathine-penicillin G procaine at 84 000 IU/kg IM at seven day intervals for three injections. However, in those with moderate to severe infections a higher dose may be required (100 000 IU/kg IM), and injection should always be facilitated with the use of a 18-gauge needle to avoid under-dosing. Treatment is recommended due to the clinical effects observed; the close correlations to the rabbit model where decreased rates of conception,

metritis and neonatal deaths were reported, and due to the potential for *Treponema* to be altering immune responses.

CHAPTER 7

Discussion

and

Conclusions

The Gilbert's potoroo is Australia's most critically endangered mammal. Its known habitat is Mount Gardner, in the Two Peoples Bay Nature Reserve with an extent of occurrence estimated to be 8km^2 and an area of occupancy estimated to be less than 5km^2. A small population of translocated individuals also exists on Bald Island, and four individuals are housed in the captive colony within the Two Peoples Bay Nature Reserve. It is possible, although unlikely, that other populations persist in some undisturbed areas along the south coast of Western Australia (Courtenay and Friend 2004).

In small populations disease can have a devastating impact, particularly in those restricted to a small range (Jacob-Hoff 1999). Genetic diversity and ability to cope with change is also imperative to species survival. Sinclair *et al.* (2002) reported a genetic bottleneck in the Gilbert's potoroo, consistent with a demographic decline. Overall, results from this study highlighted the concern for the long-term survival of this species. The Gilbert's potoroo Recovery Team was approached in 2004 to undertake a collaborative health and disease study. The research aimed to determine the prevalence of specific diseases in the wild and captive populations and to correlate the effects of identified diseases on population dynamics including reproductive success and survivorship. Diseases investigated included cryptococcosis, endoparasitism, ectoparasitism, haemoparasitism, toxoplasmosis, and *Treponema* infection. Physical examination, blood and urine collection was also undertaken to evaluate the general health of the Gilbert's potoroos and to establish reference ranges for haematology, biochemistry and urine values (Appendix 13 and 14). This information was then used to formulate population health management procedures to facilitate on-going management of the population.

From re-discovery in 1994, only eight captive-bred young have been raised. Breeding of captive individuals ceased in 2002, despite continued attempts at pairing (Friend 2005). The wild population on Mount Gardner has however remained stable in numbers. The species and its habitat are subject to a number of ongoing and potential threats including fire, predation from foxes and feral cats, low recruitment of young to the adult population, the impact of the dieback

disease on edible fungi host plants and, the clearing of areas within the species home range (Courtenay and Friend 2004). As such a novel approach was required to increase population numbers. In 2005, the first translocation to a nearby predator-free offshore island was undertaken and the results of survivability and reproduction have been encouraging. The founder population was small, a wild male and female were translocated by helicopter over to Bald Island in June 2005 (Friend 2004). Following the successful trial, these animals were retrieved and retained in captivity. Then in August 2005 these two individuals and another male and female were permanently translocated. In April 2008, seven potoroos were caught, and the first female to be born on the island was found to have pouch young. The first female taken to the island also has pouch young and a joey at heel. This was the first evidence of a second generation on the island.

These translocations occurred at the same time as the health and disease study of the captive and Mount Gardner wild populations. Baseline data has been collected and analysed, and an understanding of disease risk and current disease status of the Gilbert's potoroo has now been established. Future and widespread translocation success is dependent on this information being effectively used through the development of a quarantine period encompassing the following health screening, a review of the outcomes, risk evaluation of identified disease in light of baseline data, disease prevalence, severity of disease and the assessment of treatment against the potential stressors associated with treatment.

Pathogens can cause chronic infections and persist in individual hosts (Lyles and Dobson 1993). However it may also be argued that the translocated population size is below the threshold size and density required for epidemics of virulent pathogens to establish and persist (Lyles and Dobson 1993). The potential for closely related species to effectively increase the population size, and spill over from other reservoir hosts, could have had disastrous effects (Mathews *et al.* 2006). The most closely related species on the island is the quokka. The quokka is a small macropod with a reported disease susceptibility similar to that of the Gilbert's potoroo. No health screening

has been undertaken of these island con-specifics, and as translocated animals are particularly vulnerable given their lack of acquired immunity from previous exposure and stress induced immunosuppression the development of a health screening protocol for these individuals must also be undertaken. A review of the literature should identify the diseases of greatest concern (Jacob-Hoff 1999). Diseases and infectious agents of importance to Gilbert's potoroos should be screened for including cryptococococcosis, endoparasitism, ectoparasitism, haemoparasitism, toxoplasmosis, and *Treponema* infection. A review of outcomes and risk assessment should then occur prior to any further translocation attempts. Longitudinal monitoring is also imperative to identify the pathogens of greatest importance (Mathews *et al.* 2006).

The following recommendations are made for future translocation of all Gilbert's potoroos, based on the recommendations of Jacob-Hoff (1999) and results of health screening of the wild and captive population.

Individuals to be translocated should undergo the standard DEC morphometric analysis trap-side on Mount Gardner to collect data including body weight, body condition, presence of pouch young, ectoparasite load, and teeth wear and condition to determine age (Figure 7.1). Body weight and condition score should be evaluated and individuals below body condition score 2 should not be translocated (most wild individuals are body condition score 2). Further diagnostics and supportive care may be required for those individuals below body condition score 2. If the individual is female, with a pouch young of a critical size (in terms of its vulnerability and survival) translocation should not be undertaken to prevent the possibility of pouch young loss associated with excessive handling. If the ectoparasite load is severe (3+), with multi-focal distribution causing alopecia, skin inflammation and crusting, translocation should not be attempted until the parasitism has been treated and resolved. Teeth wear should be evaluated to age the animal and to assess its periodontal health. An aged potoroo with significant wear would not be a good candidate for translocation.

If none of the above parameters preclude translocation a faecal sample should be collected from the trap for faecal flotation and evidence of endoparasitism and any evidence of faecal soiling or gastrointestinal disease made. The potoroo should then be transferred to an individual quarantine pen in the captive colony. Appropriate shoe covers or foot baths should be set up outside each pen and there should be no mixing of food, water bowls or utensils of those under quarantine. The keepers regularly servicing the quarantine enclosures should wear a long sleeved coat or protective overalls when servicing the quarantined animals. The diet should match the current captive diet and should be weighed prior to feeding and at food bowl collection to assess food consumption. Consideration should be given for dehydration of fruit and vegetable matter altering total weights of food in particularly hot weather. The pens must all be covered to minimize access to bird guano and the potential development of cryptococcosis. The sand in the enclosures must also be clean of cat faeces. This is best achieved by removing 30cm of topsoil from a covered sand pile prior to transfer to the captive pens, in doing so minimizing the potential for toxoplasmosis. The quarantine facility should also be locked and separate quarantine waste disposal organised.

Quarantine should be instituted for 30 days to enable effective observations of illhealth and disease and to enable necessary treatments to be undertaken. It is recommended that a quarantine health screening examination under general anaesthesia is undertaken to facilitate sample collection and to minimise stress to the animal. This should occur within the first five days of capture to ensure samples can be processed and analysed within the specified quarantine period. Results of the first faecal screening should be received prior to anaesthesia to enable treatment to be undertaken under anaesthesia if required.

Through the initial health and disease study, field researchers and project leaders were shown to be competent in gaseous anaesthetic induction and maintenance using the methods outlined in chapter 2.1.2. In the absence of a veterinarian, it would be preferable for these individuals who also become competent in sample collection to undertake the required procedures as per chapter

2.1.2 and 2.1.3. However, veterinary telephone support should always be available in the event of an emergency.

Potoroos should be induced with Isoflurane (Veterinary Companies of Australia, Kings Park, NSW) delivered via face mask at 3.5% Isoflurane with an oxygen flow rate of 2.5L/minute. Five minutes post induction most potoroos should be able to be maintained on 1.5% Isoflurane and 2L/minute of oxygen. The use of the pulse oximeter will facilitate the electronic monitoring of heart rate and oxygen saturation and a stethoscope should be used to auscultate the heart for rhythm abnormalities and the lungs for respiratory noises. Anaesthetic and general physical exam data sheets (Figure 7.1) should be completed every time an individual is anaesthetised.

The procedures to be undertaken slightly differ to those described in detail in chapter 2.1.3. This follows evaluation of some of the tests undertaken and their lack of efficacy in diagnosing infection in Gilbert's potoroo. Furthermore, some testing was undertaken purely to aid in the establishment of normal baseline parameters or alternatively to further explore the pathological consequences of the disease. Cost of disease testing was also a consideration.

- A general physical examination should be undertaken. Eyes, ears, nose, oral cavity, coat condition, and feet should be checked. In particular the urogenital region and cloaca should be assessed. In males the prepuce should be examined for discharge and an assessment made according to the balanoposthitis chart (Appendix 4).

- The individual should be weighed and body condition scored to assess changes which have occurred in captivity. Feeding records should also be examined in light of these findings.

- Blood should be collected for haematology, biochemistry, and *Toxoplasma* and *Cryptococcus* serology. A blood smear should also be made for haemoparasite

examination. Results should be interpreted in light of the established reference ranges (Appendix 13).

- A dry swab from the prepuce in males and the urogenital sinus in females should be obtained for PCR for *Treponema* infection.

- A wet swab (Amie's transport medium) from the prepuce in males and the urogenital sinus in females should be obtained for dark field microscopy for spirochaetes.

- In the event of gastro-intestinal symptoms a cloacal swab should be taken for culture and results interpreted in conjunction with chapter 5 findings. Routine cloacal swabbing is not thought necessary as a baseline of commensal species and potential pathogens has now been formulated (Chapter 5.4.2, Appendix 5).

- A nasal swab for *Cryptococcus* should be taken. Owing to the lack of positive environmental samples and foot swab samples these testing procedures should only be undertaken if LCAT serology reveals a titre consistent with cryptococcosis or a nasal swab culture reveals growth and evidence of respiratory or neurological disease is present. These environmental samples would need to be taken from the trapline on which the potoroo was found.

- Urine should be collected for urinalysis and urinary glycolate testing to detect those individuals which may be familialy disposed to renal oxalosis. Cystocentesis is the preferred method of collection.

The following treatments are recommended based on results of prior faecal flotation and the health and disease study while under anaesthesia:

- Like captive Gilbert's potoroos, those to be translocated should be sprayed with a combined insecticide containing pyrethrin, N-Octyl-bicycloheptene and Piperonyl butoxide (Fido's fre-itch concentrate®, Mavlab,Queensland) and animals should be given the macrocyclic lactone Ivermectin (Ivomec® Merial, Australia) topically at 200ug/kg for ectoparasite treatment and anthelmintic endoparasitic worming. No adverse effects of this practice have been found in captive Gilbert's potoroos.

- Coccidiostats may be required if a high coccidial burden is found in conjunction with clinical signs of gastrointestinal disease. Toltrazuril (Baycox at 25mg/kg PO SID for three days), or Trimethoprim and sulfonamide combinations (Tribrissen at 40mg/kg) are recommended. Fluid therapy may also be required.

- Given the high prevalence of infection with *Treponema* all potoroos to be translocated should be treated with penicillin G benzathine-penicillin G procaine at 84 000 IU/kg IM (for those with mild clinical signs) or at 100 000 IU/kg IM (if showing moderate-severe signs) at seven day intervals for three injections. The first injection is to be given under anaesthesia. However, in those with moderate to severe infections, a higher dose may be required, and injection should always be facilitated with the use of a 18G needle to avoid under-dosing. This regime has only been trialled in long-nosed potoroos. It would be preferable to conduct further studies in captive Gilbert's potoroos prior to widespread treatment of individuals to be translocated.

A repeat faecal sample should be obtained one week following parasite treatment to check efficacy of treatment. The second and third penicillin injection will also need to be administered at weekly intervals. At these times the nature of discharge and associated urogenital signs should also be observed and the individual weighed to assess changes over the course of quarantine.

Once the samples have been analyzed a veterinarian will need to summarise the health of the individual to be translocated in light of reference ranges and disease prevalence and to decide whether the individual belongs to one of three categories outlined below (Jacob-Hoff 1999).

- Category 1 – The individual is healthy and no threat to the recipient population has been identified.
- Category 2 – The individual has shown signs of ill health and may pose a threat to the recipient population.
- Category 3 – Diagnostic tests have uncovered a disease which may pose a threat to the recipient populations.

By placing the individual in one of these three categories a decision can be made to proceed, delay or cancel the translocation. Following this, a review of the process which may include logistical issues, time delays and staff training should be undertaken to ensure continual refinement of the process and best possible outcomes for the recovery of the Gilbert's potoroo.

The main potential point of contention with this protocol is the blanket treatment of all potoroos undergoing translocation with penicillin for *Treponema* infection. It is difficult to comment on the population reproductive effects of *Treponema* infection in the Gilbert's potoroo, as both the wild and captive population are infected, therefore there is no control group for comparison. Data analysis revealed that four infected adult females with *Treponema* infection are still reproducing, and one infected sub-adult female is not. The recommendation for blanket treatment is justified for the following reasons.

The point prevalence of *Treponema* infection was 68.2% (30/44) over the three year study period. This is a significant proportion of the population which is infected. Human and animal models of infection were studied, and venereal or horizontal contact in adults and vertical or horizontal transmission in sub-adult Gilbert's potoroos was thought to be most likely. These conclusions are based on case studies involving a cross-fostered pouch young that subsequently developed

Treponema infection and a non-infected member of the captive colony paired with an infected male that subsequently became infected. Although these are only two cases, these findings are significant given the estimated population size is only 35 individuals.

In rabbits it has been shown that carriers of *Treponema paraluis-cuniculi* may be asymptomatic until stress occurs (DiGiacomo *et al*. 1983). In clinical cases, Saito *et al.* (2003) found stress triggered symptoms and that severe stress may be an important predisposing factor in outbreaks. Therefore translocated animals may be particularly vulnerable to infection given the likelihood of stress-induced immunosuppression (Mathews *et al.* 2006). Cunliffe-Beamer and Fox (1981) proved that vertical transmission was thought to be capable of perpetuating spirochaetosis in an enzootically infected colony. Therefore, infected young virgin rabbits could introduce disease into an existing breeding colony. Assuming a similar mode of transmission occurs in Gilbert's potoroo then young sub-adults, PCR negative for *Treponema*, lacking spirochaetes on dark field microscopy and lacking discharge could be carrying the disease. This could then become clinical when the individual is stressed (for example upon translocation). The act of placing the individual under a 30 day quarantine may also induce a stress response which could also lead to clinical *Treponema* infection. However a quarantine period is recommended as the disease can be treated and monitored, as compared to a direct translocation where the individual may become clinically infected in their new translocated environment and then transfer infection to other individuals which may also be in a state of stress-induced immunosuppression due to environmental factors.

Figure 7.1 Gilbert's potoroo anaesthetic and general physical exam data sheets for translocation.

Checklist for translocated Gilbert's Potoroo *(Potorous gilbertii)*

Date:_____

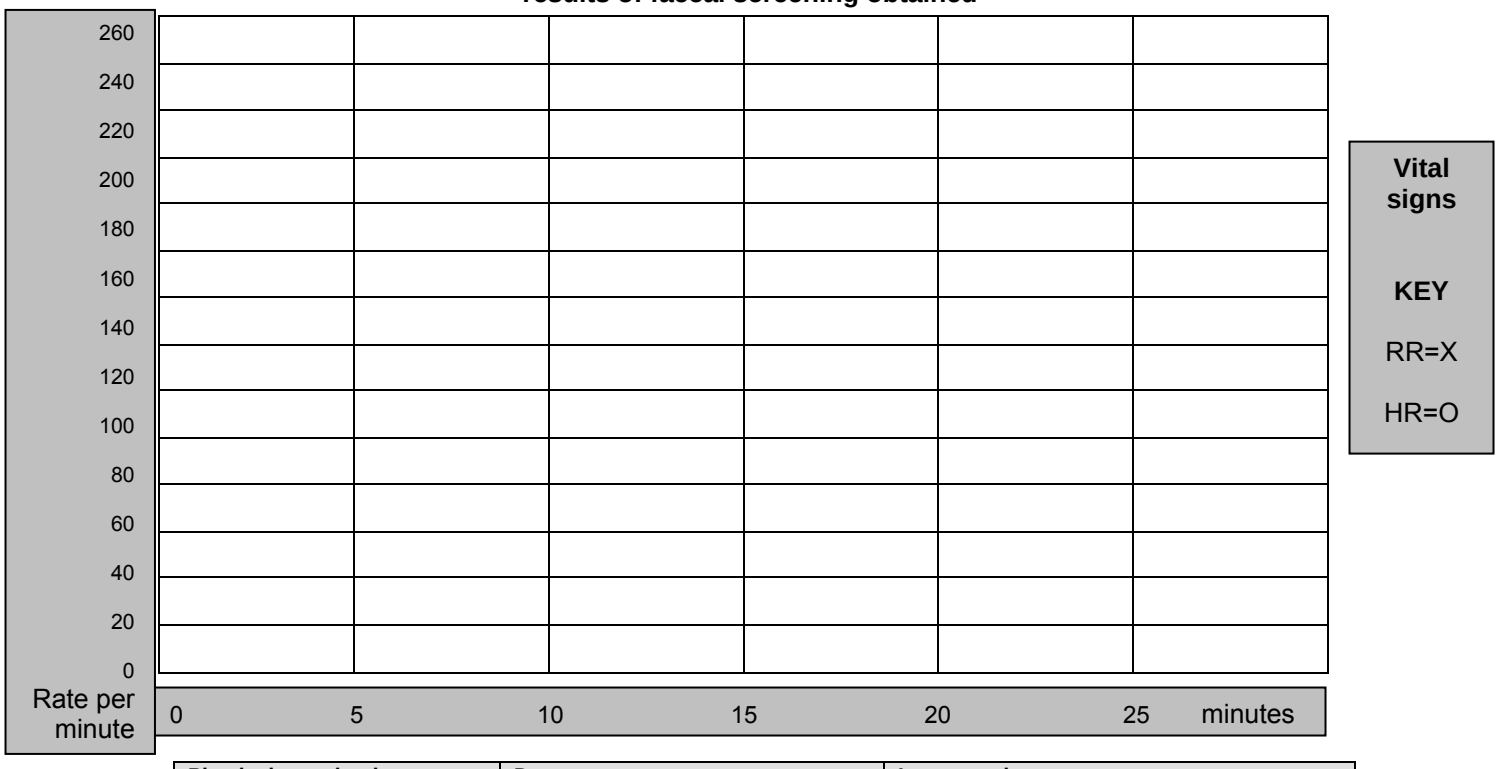
Location:_____

Capture time.....Demeanour.....ID/chip.....

Parameter	Data	Interpretation
Weight		
Body condition		1 emaciated - 5 – obese Must be over condition score 2
Presence of pouch young		Not if of 'critical size'
Teeth wear/age		Not if aged or significant teeth wear
Ectoparasites		Not if multiple sites with severe associated skin pathology

Collect faecal for endoparasite screening if above parameters OK
Transfer to captive colony and start 30 day quarantine

Within 5 days quarantine examination to be conducted under general anaesthetic with results of faecal screening obtained



Physical examination	Data	Interpretation
Weight		Compare to initial weight at trapping
Body condition		Compare to initial score at trapping
Coat condition		
Ectoparasite burden		
Ears		Check for ectoparasites,discharge
Eyes		Check for discharge, conjunctivitis
Nose		Check for depigmentation, discharge, growths
Oral cavity		Check teeth for wear and gums for inflammation
Feet		Check nail length
Urogenital region		Assess in light of balanoposthitis chart

Time of induction……End of anaesthetic……..……
Rhythm abnormalities:……..Respiratory noises……..

Sample collection based on 1kg body weight

3 mL whole blood constituting 0.5mL into EDTA & 2.5mL into serum and 1 blood smear

Test	Minimum volume ml	Tube
Haematology	0.5	EDTA
Biochemistry	0.5	Serum
Cryptococcosis LCAT	0.5	Serum
Toxoplasma DAT/MAT	0.5	Serum

Swab site	Male	Female
DRY		
Treponema for PCR	Preputial	Urogenital
WET – STUARTS MEDIUM		
Faecal culture – *if signs of gastrointestinal disease*	Cloacal	Cloacal
WET – AMIE'S MEDIUM		
Cryptococcus for culture	Nasal (dry then into Amie's)	Nasal (dry then into Amie's)
Treponema for DFM	Preputial	Urogenital

Treatment	Dose	Administered (tick box)
Pyrethrin, Piperonyl butoxide & N Octyl-bicycloheptene		
Macrocyclic lactone	Ivermectin 200 ug/kg topically	
Penicillin	84 000 IU/kg IM SIW for 3 weeks	Week 1
		Week 2
		Week 3

Follow-up faecal one week post worming to assess parasite burden

Analyse and assess all data to determine health of individual and decide on outcome

Outcome		Follow-up actions
No threat to recipient population	**Transfer**	
Individual is ill or may pose a threat to recipient population	**Delay**	
Individual has a disease which may pose a threat to the recipient population	**Cancel**	

While the disease does not cause severe debilitation, in males the presence of *Treponema* infection can be associated with a severe balanoposthitis and severe cloacal inflammation often making extrusion of the penis difficult. The clinical signs of erythema, inflammation, ulceration and a green tenacious discharge were most frequently reported and were strikingly similiar to those seen in rabbits where genital lesions contributed to reduced reproductive capabilities through dyspareunia (Cunliffe-Beamer 1981). Clinical cystitis was also suspected to be caused by *Treponema* infection in 9.1% (3/33) Gilbert's potoroos (Appendix 14). Colony outbreaks in rabbits resulted in a decreased rate of conception and increased rate of metritis, placenta retention and neonatal deaths. Furthermore the possibility of alterations in immune function increasing susceptibility to secondary disease should not be overlooked. Cunliffe-Beamer and Fox (1981) found rabbits developed severe respiratory disease due to *Pasteurella* soon after severe clinical vent disease became apparent. They suggested that *Treponema paraluis-cuniculi* may alter immune response and as such the disease should not be ignored. Similiar alterations in immune responses have also been reported in rabbits experimentally infected with the closely related *Treponema pallidum* (Shell and Musher 1974).

We have presented a strong case implicating *Treponema* as the causative agent of the observed preputial inflammatory response. All individuals that had spirochaetes on dark field microscopy had *Treponema* infection. Furthermore, histopathology revealed a moderate chronic erosive inflammatory response with diffuse, moderate to marked secondary epithelial hyperplasia in conjunction with moderate numbers of silver stained spirochaetes suggesting a strong causative relationship. The silver stained spirochaetes 2-4µm in length, were diffusely scattered over the mucosal surface and were associated with a moderate accumulation of inflammatory cells, predominantly macrophages, but also lesser lymphocytes and plasma cells. A second biopsy reported similar findings in conjunction with a low to moderate number of neutrophils forming small microabscesses within the epithelium. These histopathological changes are similar to those reported in rabbits which typically had erosions or shallow ulcers overlaid by thick crusts composed of necrotic epidermal cells and neutrophils. Epidermal microabscesses or vesicles were occasionally observed and the predominant dermal lesion was infiltration of plasma cells or

macrophages (Cunliffe-Beamer and Fox 1981). Likewise in the study of Saito *et al.* (2003) an ulcerative, lymphocytic dermatitis with necrotic foci extending from the epidermis to the dermis, with marked hyperkeratosis and silver stained spirochaetes was reported. The Treponeme found in the Gilbert's potoroo although unable to be cultured, was detected through PCR using Treponeme-specific primers, and the resulting amplicon was sequenced to enable identification of the organism within lesions. Serological tests and immunohistochemistry however were non-diagnostic.

Cunliffe-Beamer and Fox (1981) reported that venereal spirochaetosis in rabbits can be diagnosed if the causative agent, is observed, or if serological tests are positive. Although, these above findings would definitively diagnose the condition according to the rabbit model, there were a few instances where the presence of *Treponema* infection in potoroos did not definitively fit with associated inflammation. Not all individuals with clinical signs and discharge had *Treponema* infection, 5 males with moderate (2+) discharge did not have *Treponema* infection. This finding is most likely related to a false negative PCR test. However, no dark field microscopy results were obtained to validate this assumption. Twelve females with no discharge also had *Treponema* infection. *Treponema* infection in females however was not always associated with discharge therefore this finding was not unexpected. Likewise, three males were PCR negative despite having 3+ discharge and the presence of spirochaetes on dark field microscopy. Therefore the conclusion was made that clinical signs and the presence of discharge and PCR positivity is not as sensitive or specific as finding spirochaetes on dark field microscopy. These cases which may have demonstrated that *Treponema* was not part of the inflammatory response and involved in the presence of discharge, seem largely related to false negative PCR results.

The polymicrobial environment on the preputial tissue associated with the *Treponema* infection also raises questions concerning the pathogenicity of the *Treponema* infection in the Gilbert's potoroo. Many treponemes require a preceeding anaerobic micro-environment to multiply and produce disease (Hirsh 1990). *Treponema denticola* is strongly associated with the progression of adult human periodontitis and typically associated with other obligate anaerobes including

Porphyromonas gingivalis and *Bacteroides forsythus* (Edwards *et al.* 2003). Likewise in pigs *Treponema hyodysenteriae* causing colon disease must be associated with *Bacteroides vulgates*, *Bacteroides fragilis*, *Fusobacterium necrophorum*, *Campylobacter coli*, *Clostridium* spp and *Listeria denitrificans* to produce disease (Hirsh 1990). No reports of microbes associated with *Treponema paraluis- cuniculi* were found.

Overall the presence of discharge was associated with a polymicrobial environment containing *Treponema*, *Actinobacillus* and *Pasteurella*. Presence of discharge was most positively associated with *Treponema* infection, although those lacking *Treponema* infection with *Actinobacillus* infection and *Pasteurella* infection still presented with discharge. *Actinobacillus* and *Pasteurella* were cultured from all preputial biopsies from both the Gilbert's and long-nosed potoroo and odds ratios from urogenital cultures confirmed that the *Treponema* infected population was more likely to have an associated *Actinobacillus* (5.824 times) *and Pasteurella* sp. infection (3.343 times) than the non-*Treponema* infected population.

The penicillin based treatment trial in long-nosed potoroos found a continued presence of *Actinobacillus* and *Pasteurella* in all three potoroos despite clearance of *Treponema* infection in two (A20406 and A40367) and a reduction in swelling and discharge in two of three individuals (A30237 and A40367). One individual (A20406) showed no clinical signs of preputial inflammation or discharge. The continued presence of *Actinobacillus* and *Pasteurella* was thought to reflect that these two bacteria were playing a role in a polymicrobial relationship to optimise conditions for the growth of *Treponema* and were not the primary pathogens owing to the reduction in clinical symptoms seen in the absence of *Treponema* infection. The increased magnitude of *Actinobacillus* and *Pasteurella* in the persistently *Treponema* infected long-nosed potoroo compared to those that had cleared the infection confirmed this statement. The *Actinobacillus* and *Pasteurella* flourished and in doing so optimised conditions for continued *Treponema* growth. The penicillin dosage in this individual was thought to be below the requirement.

Blanket treatment of potoroos is therefore recommended due to the severity of clinical effects observed; the close correlations to the rabbit model in terms of clinical signs and histopathology and owing to the reported findings of decreased rates or conception, metritis and neonatal deaths in infected rabbit colonies; the possibility of the stress of translocation exacerbating disease and leading to clinical infection, the potential for venereal, direct, horizontal and direct transmission propagating infection, and the potential for *Treponema* to be altering immune responses.

There has been a lot of discussion regarding immune function and the potential of stress induced immunosuppression affecting translocated individuals (Saito *et al.* 2003; Mathews *et al.* 2006). Constraints in blood volume collection due to body size and other disease testing requirements as well as logistical constraints with the diagnostic immune function testing laboratory being situated at Macquarie University in Sydney, and shipping time, meant immune function testing via mitogen (PHA) driven proliferation assays (Ashman *et al.* 1976; Brozek *et al.* 1992: Buddle *et al.* 1992; Wilkinson *et al.* 1992) was not able to be conducted in this health and disease study. However immune function testing of this species would certainly be warranted if the above constraints were not an issue.

No virology was undertaken in this study owing to the lack of laboratories in Australia screening for viral diseases of interest, including herpesvirus, orbiviruses and encephalomyocarditis virus. Herpes virus is the virus of greatest interest as conjunctivitis, pyrexia, respiratory distress and death have been reported in infected macropods (Blyde 1999). Ulceration of the genital tract, especially mucocutaneous blistering of the penis and cloaca, has also been reported in macropods associated with this disease (Munday 1988). Currently definitive diagnosis is based on finding intra-nuclear inclusion bodies in hepatocytes from histopathology (Blyde 1999). However, if non-invasive testing became available, and a review of the current literature justified screening, then herpes virus screening should also be included in the translocation health and disease protocol.

A field trip to Bald Island in February 2008 revealed that all of the translocated males have green preputial discharge (T. Friend personal communication, February 2008). To effectively eradicate *Treponema* infection all exposed translocated individuals must be treated, this includes pouch young. Treating only clinically infected individuals in rabbits did not eradicate infection from an enzootically infected colony (Cunliffe-Beamer and Fox 1981). Therefore it is recommended that the entire translocated population on Bald Island be captured for penicillin treatment once a week for three weeks. No further individuals should be translocated until this has been undertaken.

This thesis has established baseline blood, urine and microbiology information and has identified diseases of significance to the Gilbert's potoroo. Existing knowledge from wildlife health, human health, epidemiology, ecology, conservation biology and veterinary science have been combined to develop a solution-oriented approach to minimize the impact of disease on the existing Gilbert's potoroo population. A checklist for translocation procedures, including quarantine health and disease screening and treatment of identified significant diseases has been formulated to aid in the recovery program of Australia's most critically endangered mammal. This protocol however only forms the first step in the development of a full disease screening program. It will not be effective on its own. To ensure efficacy, it must be implemented with a schedule of longitudinal individual sampling of both Gilbert's potoroo and con-specific species, particularly those resident on Bald Island. While health screening of the translocated population may present logistical challenges due to the terrain and lack of settlement and facilities on Bald Island, the potential for the early identification of emerging disease or increased pathogen load may prove crucial to the species survival of Gilbert's potoroo, Australia's most critically endangered mammal.

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APPENDIX 1: Gilbert's potoroo anaesthetic and general physical exam data sheets.

Physical examination checklist for Gilbert's Potoroo *(Potorous gilbertii)*

Date:_____

Location:_____

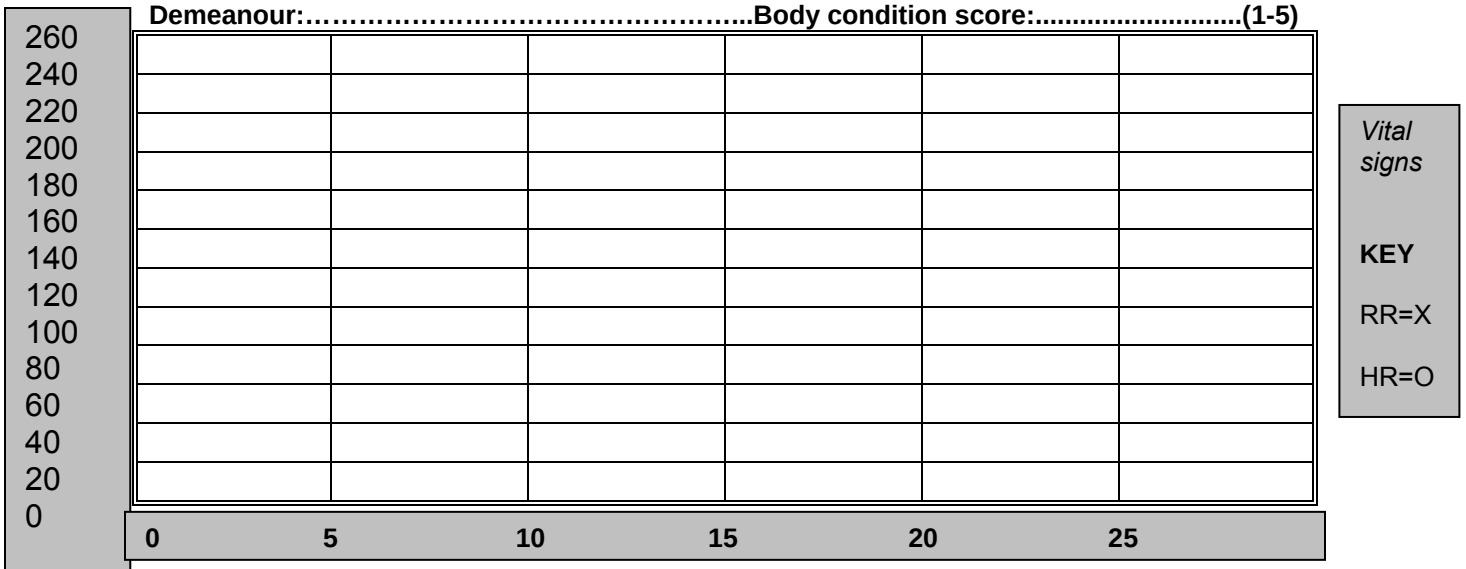
Faecal sample: ☐ **Fresh** ☐ **From trap** ☐ **From bag**

Capture time.....**Sex**.....

ID/chip.....**Weight**.....

Time of induction:……**ET Tube size:**……..

Demeanour:……..**Body condition score:**.....**(1-5)**



Haircoat:……

Integument:……

Skin scrapings ☐ **paraffin** ☐

EENT

Ears: ☐ **NAD** ☐ **abnormal:**……

Eyes: ☐ **NAD** ☐ **abnormal:**……

Oral cavity: ☐ **NAD** ☐ **abnormal:**…… ……

……

……

Dental Exam: ☐ **NAD** ☐ **abnormal:**……

……

……

Cardiovascular Exam

HR:……..**Rhythm abnormalities:**……..

Musculoskeletal Exam

☐ **NAD** ☐ **abnormal:**……..

……

……

Reproductive/Urogenital Exam

☐ **NAD** ☐ **abnormal:……...**
……
……

Blood collection ☐
Site:……volume:……...
EDTA: 0.5mL ☐ **Whole blood** ☐ **……**

Urine collection:

☐ **NAD** ☐ **abnormal:……..**
……
……

Swabs:
Cloacal swab: ☐ ***Culture*** ☐ ***Treponema*-like organism**
Nasal swab: ☐
Preputial swab: ☐ ***Treponema*-like organism**

Recovery time:……Alert:……
Comments:……

EMERGENCY DRUGS & DOSAGES BASED ON AN ESTIMATED 1kg BODY WEIGHT.

Drug	Formulation	Dose rate	Volume	Route
Adrenaline	1:1000 vials 1mg/mL	0.01	0.01	IV
Lignocaine	100	0.5	0.05	IV
Dopram	20mg/mL	1mg/kg IV	0.05mL	Neonate 1-5mg SC/sublingual
Atropine	0.6	0.03		IV/SC

Other drugs administered: ……
SAMPLE COLLECTION based on 1kg body weight
4mL whole blood constituting 0.5ml into EDTA & 3.5mL into serum and 1 blood smear

Test	Minimum volume mL	Tube
Biochemistry	0.5	Serum
Cryptococcosis	0.5	Serum
Haematology	0.5	EDTA
Toxoplasma DAT/MAT	0.5	Serum
* Treponema serology (March 2007 only)	0.5	Serum

Swab site	Male	Female
DRY (1)		
Treponema (1)	Preputial	Urogenital
WET – STUARTS (2)		
Faecal culture – *Salmonella*	Cloacal	Cloacal
Culture	Preputial	Urogenital
WET – AMIE'S (2)		
Cryptococcus (2)	Feet	Feet
	Nasal (dry then into Amie's)	Nasal (dry then into Amie's)

APPENDIX 2: Gilbert's potoroo samples tested and dispatched.

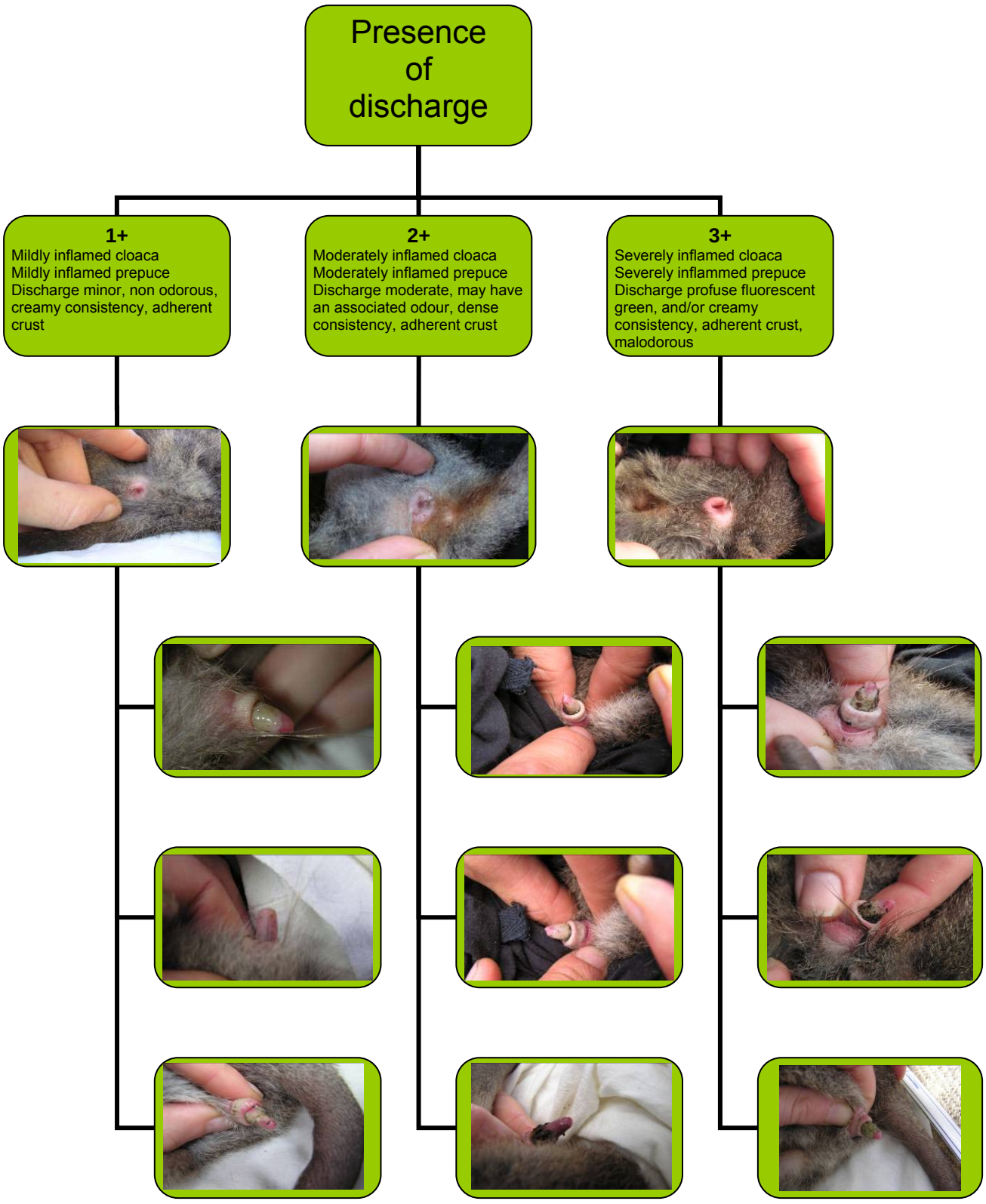
Test	Dispatched to
Blood in EDTA for haematology	**Dr Phil Clark** Murdoch University Clinical Pathology Department
Blood smear for haemoparasite examination	**Dr Phil Clark** Murdoch University Clinical Pathology Department
Cloacal swabs for bacteriology, and *Salmonella* culture if present	**Gary Allen** Murdoch University Clinical Pathology Department
Ectoparasites for identification	**Yazid Abdad** Murdoch University Parasitology Department
Faecal samples for parasite identification and faecal flotation	**Dr Russell Hobbs** Murdoch University Parasitology Department
* Preputial biopsies for pathology (November 2006 and March 2007 only)	**Dr Graeme Knowles** Murdoch University Pathology Department
Preputial and urogenital swabs for *Treponema* PCR	**Dr Nicky Buller** Department of Agriculture and Food WA
Whole blood for biochemistry	**Dr Phil Clark** Murdoch University Clinical Pathology Department
1) Whole blood for LCAT serology 2) Feet and nasal swabs for *Cryptococcus* culture	**Dr Mark Krockenberger** University of Sydney
* Whole blood for *Treponema* serology (March 2007 only)	**Dr Peter Phillips** PathWest WA State Syphilis Research Laboratory
Whole blood for *Toxoplasma* DAT/MAT serology	**Gary Allen** Murdoch University Clinical Pathology Department then to Australian Animal Health Laboratories in Tasmania
Urogenital swabs for microbial culture	**Dr Nicky Buller** Department of Agriculture and Food WA
Urine for urinalysis and culture	**Gary Allen** Murdoch University Clinical Pathology Department

APPENDIX 3: Total population sampled for gastro-intestinal parasites.

Gilbert's Potoroo ID	May-05	Jun-05	Nov-05	Jun-06	Nov-06	Total number of faecal samples collected
M28[A]	X					1
M46	X	X	X	X	X	5
M49[B]	X					1
F50			X		X	2
F54	X					1
M55		X	X	X		3
F57	X	X	X	X		4
F61			X			1
M65	X	X				2
F66		X				1
M68	X	X	X	X		4
F69			X	X	X	3
M70			X			1
M72		X				1
M77	X	X				2
F78	X			X		2
M83		X	X	X	X	4
F86	X	X				2
F89	X	X	X			3
F92				X		1
F93	X	X	X	X	X	5
M94	X		X	X	X	4
F98		X				1
F100			X			1
F101		X				1
M102		X	X			2
F103			X			1
M104			X			1
F106			X			1
M107			X	X	X	3
M110				X		1
M111				X		1
M115				X		1
M116					X	1
F119					X	1
F120					X	1
M123					X	1
TOTAL	**13**	**15**	**18**	**14**	**10**	**71**
WILD	**9**	**10**	**14**	**10**	**7**	**51**
CAPTIVE	**4**	**5**	**4**	**4**	**3**	**20**

[A] captive Gilbert's potoroo (n=7) in bold font
[B] wild Gilbert's potoroo (n=30) in regular font

APPENDIX 4: Chart grading the severity of balanoposthitis in Gilbert's potoroos.



APPENDIX 5: Seasonal microbiology findings in the rectal orifice of the cloaca in the Gilbert's potoroo population.

Microbes found in Gilbert's potoroos	March 2005 (n = 13)	June 2005 (n = 15)	November 2005 (n = 18)	June 2006 (n = 15)	November 2006 (n = 12)	Number of sessions bacteria found
Bacillus spp.	2/19 10.5%			2/26 7.7%	2/17 11.8%	**3/5** **60%**
Coryneform bacilli	2/19 10.5%	2/11 18.2%	3/27 11.1%	6/26 23.1%	2/17 11.8%	**5/5** **100%**
Enterococcus sp.	2/19 10.5%	3/11 27.3%	4/27 14.8%		3/17 17.6%	**4/5** **80%**
E .coli	3/19 15.8%	3/11 27.3%	6/27 22.2%	6/26 23.1%	5/17 29.4%	**5/5** **100%**
Pasteurella	2/19% 10.5%			1/26 3.8%		**2/5** **40%**
Proteus mirabilis	1/19% 5.3%		2/27 7.4%	2/26 7.7%		**3/5** **60%**
Pseudomonas spp.	1/19 5.3%		1/27 3.7%	1/26 3.8%		**3/5** **60%**
Serratia liquefaciens			1/27 3.7%		1/17 5.9%	**2/5** **40%**
Staphylococcus sp. *coagulase +*	1/19 5.3%			1/26 3.8%		**2/5** **40%**
Staphylococcus sp. *coagulase -*			1/27 3.7%		1/17 5.9%	**2/5** **40%**
Streptococcus sp. *α-haemolytic*	2/19 10.5%	3/11 27.3%	5/27 18.5%	4/26 15.4%	1/17 5.9%	**5/5** **100%**
Streptococcus sp. *non-haemolytic*			4/27 14.8%	2/26 7.7%	2/17 11.8%	**3/5** **60%**
Streptococcus sp. *β-haemolytic*				1/26 3.8%		**1/5** **20%**
Number of species in total	**9**	**4**	**9**	**10**	**8**	
Number of isolates in total	**19**	**11**	**27**	**26**	**17**	

APPENDIX 6: Total population from which urogenital swabs were collected.

Gilbert's Potoroo ID	May-05	Jun-05	Nov-05	Jun-06	Nov-06	Mar-07	TOTAL number of sessions sampled
M28	X	X	X				3
M46	X	X	X	X	X	X	6
M49	X						1
F50			X		X	X	3
F54	X						1
M55		X	X		X	X	4
F57	X	X	X	X	X	X	6
F61			X			X	2
M65	X						1
F66		X	X				2
M68	X	X	X	X	X	X	6
F69			X			X	2
M70			X				1
M72		X					1
M77		X					1
F78	X						1
M83		X	X	X	X	X	5
F86	X					X	2
F89	X		X			X	3
F92				X	X	X	3
F93	X	X	X	X	X	X	6
M94	X	X	X	X	X	X	6
F98	X	X					2
F100			X				1
F101		X					1
M102		X	X				2
F103			X			X	2
M104			X				1
F106			X				1
M107			X	X	X	X	4
M111				X			1
F112				X			1
M113						X	1
M116				X	X	X	3
F119					X	X	2
F120					X	X	2
M121						X	1
M126						X	1
F128						X	1
TOTAL	**13**	**14**	**20**	**11**	**13**	**22**	**39**
WILD	**9**	**8**	**14**	**7**	**9**	**17**	**31**
CAPTIVE	**4**	**6**	**6**	**4**	**4**	**5**	**8**

APPENDIX 7: Seasonal aerobic urogenital swab results.

Aerobic microbes isolated from Gilbert's potoroos	March 2005 (n = 13)	June 2005 (n=14)	November 2005 (n = 20)	June 2006 (n = 11)	November 2006 (n = 13)	March 2007 (n=22)	Number of sessions bacteria found
***Actinobacillus* sp.**	1/11 9.1%	1/30 3.3%	11/41 26.8%	4/33 12.1%	7/40 17.5%	13/53 24.5%	**6/6 100%**
***Bacillus* spp.**		1/30 3.3%	1/41 2.4%		2/40 5%	1/53 1.9%	**4/6 66.6%**
Brackiella oedipus	1/11 9.1%		7/41 17.1%	3/33 9.1%	7/40 17.5%	8/53 15.1%	**5/6 83.3%**
Coliforms	1/11 9.1%			1/33 3%			**2/6 33.3%**
***Corynebacterium pilosum*.**	2/11 18.2%	5/30 16.7%	8/41 19.5%	5/33 15.1%	4/40 10%	4/53 7.5%	**6/6 100%**
Enterobacter aerogenes	1/11 9.1%						**1/6 16.7%**
Enterobacter cloacae		1/30 3.3%		1/33 3%			**2/6 33.3%**
Enterobacter faecalis				2/33 6.1%	3/40 7.5%	5/53 9.4%	**3/6 50%**
Enterobacter vulneris	1/11 9.1%						**1/6 16.7%**
E. coli (haemolytic)					1/40 2.5%		**1/6 16.7%**
E. coli (non-haemolytic)		3/30 10%		2/33 6.1%	2/40 5%	8/53 15.1%	**4/6 66.7%**
Klebsiella oxytoca		1/30 3.3%			3/40 7.5%		**2/6 33.3%**
Lactobacillus				1/33 3%			**1/6 16.7%**
Non-fermentive GNR		5/30 16.7%		2/33 6.1%	2/40 5%		**3/6 50%**
***Pantoea* sp.**		1/30 3.3%				1/53 1.9%	**2/6 33.3%**
***Pasteurella* sp.**	4/11 36.4%	6/30 20%	12/41 29.3%	9/33 27.3%	5/40 12.5%	12/53 22.6%	**6/6 100%**
Proteus vulgaris		1/30 3.3%					**1/6 16.7%**
Pseudomonas aeruginosa		1/30 3.3%	1/41 2.4%	1/33 3%		1/53 1.9%	**4/6 66.7%**
Serratia marcescens				1/33 3%	1/40 2.5%		**2/6 66.7%**
Serratia liquefaciens				1/33 3%			**1/6 16.7%**
***Staphylococcus* sp.**	1/11 9.1%	1/30 3.3%		1/33 3%	2/40 5%		**4/6 66.7%**
Staphylococcus aureus		3/30 10%	1/41 2.4%		1/40 2.5%		**3/6 50%**
Staphylococcus epidermidis				1/33 3%			**1/6 16.7%**
Number of species in total	**8**	**13**	**7**	**15**	**13**	**9**	
Number of isolates in total	**11**	**30**	**41**	**33**	**39**	**53**	

GNR = Gram-negative rod

APPENDIX 8: Seasonal anaerobic urogenital swab results.

Anaerobic bacteria Isolated from Gilbert's potoroos	March 2005 (n = 13)	June 2005 (n =14)	November 2005 (n = 20)	June 2006 (n =11)	November 2006 (n = 13)	March 2007 (n=22)	Number of sessions bacteria found
Bacteroides fragilis					1/20 5%		**1/6** **16.7%**
Bacteroides melaninogenicus		1/13 7.7%					**1/6** **16.7%**
Bacteroides thetaiotaomicron	2/11 18.2%						**1/6** **16.7%**
***Bifidobacterium* sp.**						1/18 5.5%	**1/6** **16.7%**
Clostridium glycolicum		1/13 7.7%	1/22 4.5%	3/24 12.5%	2/20 10%%		**4/6** **66.7%**
Eubacterium lentum				2/24 8.3%			**1/6** **16.7%**
***Fusobacterium* sp.**						1/18 5.5%	**1/6** **16.7%**
Helcococcus	1/11 9.1%						**1/6** **16.7%**
Porphyromonas asaccharolytica		1/13 7.7%	4/22 18.2%	2/24 8.3%	6/20 30%		**4/6** **66.7%**
Prevotella	1/11 9.1%		1/22 4.5%	6/24 25%	4/20 20%	3/18 16.7%	**5/6** **83.3%**
***Treponema* – infected**	7/11 63.6%	10/13 76.9%	16/22 72.7%	11/24 45.8%	7/20 35%	13/18 72.2%	**6/6** **100%**
Number of species in total	**4**	**4**	**4**	**5**	**5**	**4**	
Number of isolates in total	**11**	**13**	**22**	**24**	**20**	**18**	

APPENDIX 9: Most commonly isolated urogenital aerobes and anaerobes

Aerobic & anaerobic microbes isolated from Gilbert's potoroos	March 2005 (n = 13)		June 2005 (n = 14)		November 2005 (n = 20)		June 2006 (n = 11)		November 2006 (n = 13)		March 2007 (n=22)		Number of sessions bacteria found
Sex affected	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	
n =	**6**	**7**	**9**	**5**	**10**	**10**	**7**	**4**	**7**	**6**	**10**	**12**	
***Actinobacillus* sp.**	1/10 10%		1/19 5.3%		9/32 28.1%	2/15 13.3%	4/24 16.7%		5/17 29.4%	2/6 33.3%	10/29 34.5%	3/13 23%	**6/6 100%**
***Corynebacterium pilosum*.**	1/10 10%	1/4 25%	4/19 21.1%	1/3 33.3%	5/32 15.6%	3/15 20%	3/24 12.5%	2/5 40%	2/17 11.7%	2/6 33.3%	1/29 3.4%%	3/13 23%	**6/6 100%**
***Pasteurella* sp.**	3/10 30%	1/4 25%	5/19 26.3%	1/3 33.3%	9/32 28.1%	3/15 20%	8/24 33.3%	1/5 20%	4/17 23.5%	1/6 16.7%	9/29 31%	3/13 23%	**6/6 100%**
Treponema	5/10 50%	2/4 50%	9/19 47.4%	1/3 33.3%	9/32 28.1%	7/15 46.6%	9/24 37.5%	2/5 40%	6/17 35.3%	1/6 16.7%	9/29 31%	4/13 30.7%	**6/6 100%**
Number of species in total	**4**	**3**	**4**	**3**	**4**	**4**	**4**	**3**	**4**	**4**	**4**	**4**	
Number of isolates in total	**5**	**4**	**19**	**3**	**32**	**15**	**24**	**5**	**17**	**6**	**29**	**13**	

APPENDIX 10: Seasonal urogenital aerobic and anaerobic microbiology findings in the *Treponema* infected compared to the non-*Treponema* infected population.

Aerobic microbes isolated from Gilbert's potoroos	No. of sessions bacteria found in total population	No. of sessions bacteria found in Treponema infected population	No. of sessions bacteria found in non-infected Treponema	Anaerobic bacteria Isolated from Gilbert's potoroos	No. of sessions bacteria found in total population	No. of sessions bacteria found in Treponema infected population	No. of sessions bacteria found in non-infected Treponema population
***Actinobacillus* sp.**	6/6 100%	6/6 100%	4/6 66.7%	***Bacteroides fragilis***	1/6 16.7%	1/6 16.7%	0
***Bacillus* sp.**	4/6 66.6%	2/6 33.3%	2/6 33.3%	***Bacteroides melaninogenicus***	1/6 16.7%	1/6 16.7%	0
Brackiella oedipus	5/6 83.3%	4/6 66.6%	4/6 66.6%	***Bacteroides thetaiotaomicron***	1/6 16.7%	1/6 16.7%	0
Coliforms	2/6 33.3%	2/6 33.3%	1/6 16.7%	***Bifidobacterium* sp.**	1/6 16.7%	0	1/6 16.7%
Corynebacterium pilosum.	6/6 100%	6/6 100%	5/6 83.3%	***Clostridium glycolicum***	4/6 66.6%	4/6 66.6%	2/6 33.3%
Enterobacter aerogenes	1/6 16.7%	1/6 16.7%	0	***Eubacterium lentum***	1/6 16.7%	1/6 16.7%	1/6 16.7%
Enterobacter cloacae	2/6 33.3%	1/6 16.7%	1/6 16.7%	***Fusobacterium* sp.**	1/6 16.7%	1/6 16.7%	0
Enterobacter faecalis	3/6 50%	1/6 16.7%	3/6 50%	***Helcoccus***	1/6 16.7%	1/6 16.7%	0
Enterobacter vulneris	1/6 16.7%	1/6 16.7%	0	***Porphyromonas asaccharolytica***	3/6 50%	3/6 50%	3/6 50%
E.coli (haemolytic)	1/6 16.7%	0	1/6 16.7%	***Prevotella***	5/6 83.3%	5/6 83.3%	2/6 33.3%
E.coli (non-haemolytic)	4/6 66.6%	3/6 50	4/6 66.7%	***Treponema* – infected**	6/6 100%	6/6 100%	0
Klebsiella oxytoca	3/6 50%	2/6 33.3%	1/6 16.7%				
Lactobacillus	1/6 16.7%	0	1/6 16.7%				
Non-fermentive GNR	3/6 50%	2/6 33.3%	1/6 16.7%				
Pantoea sp.	2/6 33.3%	1/6 16.7%					
***Pasteurella* sp.**	6/6 100%	6/6 100%	5/6 83.3%				
Proteus vulgaris	1/6 16.7%	0	1/6 16.7%				
Pseudomonas aeruginosa	4/6 66.7%	2/6 33.3%	2/6 33.3%				
Serratia marcescens	2/6 66.7%	2/6 66.7%	0				
Serratia liquefaciens	1/6 16.7%	1/6 16.7%	0				
***Staphylococcus* sp.**	4/6 66.7%	4/6 66.7%	3/6 50%				
Staphylococcus aureus	3/6 50%	2/6 33.3%	1/6 16.7%				
Staphylococcus epidermidis	1/6 16.7%	0	1/6 16.7%				

APPENDIX 11: *Treponema* infection status in individuals.

Potoroo ID n=44	Mar-05	Jun-05	Nov-05	Jun-06	Nov-06	Mar-07	Neg	Cleared	Persistent
M28	Pos	Pos	Pos						Persistent
M46	Pos	Pos	Pos	Pos	Pos	Pos			Persistent
M49	Pos								
F50			Pos (py)		Neg				
M55		Pos	Pos	Pos	Pos	Pos			Persistent
F57	Pos	Pos	Pos	Pos	Neg	Neg		Cleared	
F61			Pos			Neg			
F66		Pos	Pos						
M68	Pos	Pos	Pos	Pos		Pos			Persistent
F69			Pos (py)		Neg (py)	Neg (py)		Cleared	
M70			Pos						
M72		Pos							
M77	Pos	Neg							
F78	Pos			Neg (py)					
M83		Neg	Pos	Pos	Pos	Pos			Persistent
F86					Neg	Neg	Neg		
F89	Neg		Pos (py)			Neg (py)			
F92				Neg	Neg (py)	Pos (no py)			
F93	Neg		Pos	Pos	Pos	Pos			Persistent
M94	Neg	Pos	Pos	Pos	Pos	Pos			Persistent
M98	Neg	Neg					Neg		
F100			Pos (py)			Pos (no py)			
F101		Neg							
M102		Pos	Pos						
M103			Neg			Pos			
M104			Pos						
F106			Neg (py)						
M107					Neg	Pos			
F108			Neg						
M110				Pos					
M111				Pos					
F112				Neg					
M113						Pos			
M114				Pos					
F115				Neg					
M116				Pos	Pos	Pos			
F118						Pos			
F119						Neg			
F120						Neg			
M121						Neg			
M123					Pos				
M124						Neg			
M126						Neg			
F128						Neg			

APPENDIX 12: Sensitivity and specificity of clinical signs and the presence of discharge, dark field microscopy for spirochaetes and PCR to detect *Treponema* infection.

Diagnostic test	Sensitivity	Specificity
Clinical signs and presence of discharge	91.2%	27.9%
Dark field microscopy for spirochaetes	100%	-
PCR	95.3%	100%

APPENDIX 13: Haematological and serum biochemical reference values and cohort analysis in the Gilbert's potoroo (*Potorous gilbertii).*

In press – accepted for publication by the Journal of Zoo and Wildlife Medicine March 17 2008.

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Abstract

Haematology and serum biochemistry blood values are tabulated for Australia's most critically endangered mammal, the Gilbert's potoroo *Potorous gilbertii*. Significant differences were found between origin (captive or wild individuals) and age (sub-adult or adult). Sex and presence or absence of *Treponema* infection had minimal significance on blood values. Typical cell morphology is discussed, and haemoparasite examination identified *Theileria* spp. and *Breinlia* spp. Disparity amongst standard haematological and biochemical markers for 'stress' were found, warranting further investigation of the typical glucocorticoid response in marsupials. These reference ranges and findings will assist in the ongoing health management of this critically endangered species.

Key words: Haematology, biochemistry, marsupial, Gilbert's potoroo, *Potorous gilbertii*

Introduction

The Gilbert's potoroo *(Potorous gilbertii*) is a small marsupial endemic to the Two Peoples Bay Nature Reserve in the south-west of Western Australia. The Gilbert's potoroo was presumed to be extinct, as there had been no sightings reported after 1870. However, in 1994 a small population was rediscovered at Mount Gardner, in the Two Peoples Bay Nature Reserve near Albany. This remnant population is restricted to an 1800 ha region of heath land within the reserve. An interim recovery plan was prepared for the species immediately after its rediscovery recommending a captive breeding program be established as part of a comprehensive recovery program to insure against the catastrophic loss of the wild population (e.g. through wildfire) and, to breed individuals for translocation to establish new populations.[6] Eight animals were removed from the wild to establish the founder group for the captive breeding program. According to IUCN criteria[11] the Gilbert's potoroo is classified as Critically Endangered.[6] The current population estimate of the Gilbert's potoroo is 35 individuals (J.A. Friend, personal communication, January 2008).

A collaborative health and disease study of the Gilbert's potoroo was undertaken involving the Department of Environment and Conservation (DEC), the Department of Food and Agriculture (DAFWA) and the Perth Zoo. 80 blood samples were obtained over a three year period from 35 Gilbert's potoroos. Blood was collected to establish reference haematological and serum biochemical values, to identify effects of disease on haematological and serum biochemical values, and to explore factors which may significantly alter haematological and serum biochemical values in the Gilbert's potoroo.

Over these three years, six, three week trapping sessions were attended. Many of the captive individuals were re-trapped over the six trapping sessions. However, given there was a minimum of three months between sampling, the use of these repeated samples in the population data was thought justifiable. Overall, the number of blood samples collected differed from the number of individuals trapped. If a female had presence of pouch young which were thought to be of 'critical size' in relation to vulnerability and survivability then general anaesthesia for blood sample collection was not undertaken. Although this may place some bias on the population studied, only four females belonged to this category, and it was thought the relative risk of anaesthesia for blood sample collection far outweighed the value of assessing blood parameters for this cohort of Australia's most critically endangered mammal.

Materials and methods

Wild potoroos were trapped three times per year (March, June and November) by Department of Environment and Conservation staff to monitor the population in accordance with the Gilbert's Potoroo Recovery Plan ratified by the Gilbert's Potoroo Recovery Team. On Mount Gardner, Sheffield cage traps, were set the afternoon prior to trapping and checked at dawn. When a potoroo was found, it was transferred to a black cotton bag and a total weight obtained with the use of spring scales. Captive Gilbert's potoroos were captured using a shade cloth run and net on a fortnightly basis to enable regular weighing and physical examination.
To undertake the procedures required for disease testing the potoroo's nose was exteriorized from the black cotton bag and the animal was induced with Isoflurane (Veterinary Companies of Australia, Kings Park, NSW, 2148, Australia) delivered via face mask at 3.5% Isoflurane with an oxygen flow rate of 2.5L/minute. Five minutes post-induction most potoroos were able to be maintained on 1.5% Isoflurane and 2L/minute of oxygen.

A patch of hair overlying the left femoral triangle was clipped with WAHL® clippers (Wahl Clipper Corporation and Unity Agencies, Victoria, 3180, Australia) and the skin prepared with a combined 50/50 chlorhexidine gluconate and 70% ethanol mixture. A 23G needle and a 3mL syringe were used to obtain a blood sample from the femoral vein. Up to 0.5% of body weight in a healthy individual was recommended by P. Clark (personal communication, May 2005). The blood obtained was divided for different test procedures. A blood smear was made via the spreader slide technique for differential leukocyte and platelet counts, assessment of platelet, WBC and RBC morphology and haemoparasite examination. Approximately 0.5mL of blood was preserved in a 1.5mL EDTA tube and sent with the blood smear to the Murdoch University Department of Veterinary Clinical Pathology for a complete blood count . A further 3.5mL of blood was collected and placed into a plain tube, and later centrifuged to obtain serum. Of this total sample, 0.5mL of serum was submitted for biochemistry and 0.5mL of serum was submitted for DAT and MAT testing for Toxoplasmosis, to Murdoch University. A further 0.5mL of serum was submitted for the Latex Cryptococcal Antigen test (LCAT) for Cryptococcosis to the University of Sydney and 0.5mL of serum was submitted for the *Treponema pallidum* particle agglutination (TPPA), Enzyme immunoassay (EIA), as well as the non-specific Rapid plasmid reagin (RPR) test for detection of *Treponema* to Pathwest, Nedlands, Western Australia. These samples were sent via overnight courier on ice. Any excess serum collected was frozen at -20ºC for two days then transferred to a -80ºC freezer for future use. Urine was collected mid-stream free catch for urinalysis and forwarded via overnight courier on ice to Murdoch University.

Complete blood counts were performed on EDTA-preserved whole blood using an Advia120 hematology analyzer (Bayer, Tarrytown, New York, 10591, USA). Red blood cell (RBC) count, white blood cell (WBC) count, haemoglobin (Hb), packed cell volume (PCV), mean cell volume (MCV), mean cell haemoglobin (MCH), and mean cell haemoglobin concentration (MCHC) were measured. Blood films were stained with Wright and Giemsa stains and differential leukocyte counts, RBC and WBC morphology and haemoparasite examination was conducted under light microscopy. Fibrinogen concentration was determined using the heat precipitation method. Serum was analysed using a Randox RX Daytona analyzer (Randox Laboratories Ltd, Belfast, BT3 9HA Ireland). Alkaline phosphatase (ALP), aspartate amino transferase (AST), alanine transaminase (ALT), creatinine kinase (CK), total bilirubin, urea, creatinine, cholesterol, calcium, phosphorous, glucose, albumin, globulin and total serum protein were analysed. Total protein was measured using the Daytona (Randox Laboratories Ltd, Belfast, BT3 9HA Ireland) Biuret Reagent method, and urea was measured using the Daytona (Randox Laboratories Ltd, Belfast, BT3 9HA Ireland) Enzymatic Kinetic Method. Urinalysis was performed using Multistix® Reagent strips (Bayer, Pymble, Australia) and urine specific gravity measured through the use of a refractometer.

Data was recorded using an Excel spreadsheet (Microsoft Corporation, North Ryde, NSW, 2113, Australia) and an assessment of blood parameters made in light of physical examination findings, recovery post-procedure and the absence of any history of current or chronic illness. Four individuals were deemed unhealthy, and excluded from the study. Captive Gilbert's potoroo (GP) F66 an azotaemic, aged potoroo, with elevated BUN 26.6mmol/L (mean: 5.946 S.D: 1.5036), creatinine 334µmol/L (mean: 49.52 S.D 13.043), and clinical evidence of poluria and polydipsia. These findings were consistent with a diagnosis of chronic renal disease, which was later confirmed via histopathology following this animal's death. Captive GP F57 had a prolonged and stressful catch up in the November 2005 trapping session. This female had a resultant highly elevated CK >20 000U/L (mean 1074.4, SD 924.29) and AST 1161U/L (mean 84.64, S.D 43.37) indicative of acute muscle damage thought to be associated with the traumatic catch up as no increase in liver enzymes was apparent. Wild female GP F61 had a severe eosinophilia of 15% (mean 2.73, S.D 1.42) and a total count of $0.59x10^9$/L (mean 0.08, S.D 0.06) in March 2007. The reason behind this value is unknown, other haematology results were unremarkable and a minimal ectoparasite burden was present. Parasitism is a common cause of elevation of eosinophils in the peripheral circulation.[25] As such, this value was classed as an outlier and the possibility of laboratory error could not be ruled out. The final individual was aged, wild GP M55 who was in poor body condition (condition score 1.5), had pale mucous membranes, a prolonged recovery from anesthesia and haematological evidence of anaemia in July 2005. HCT 0.16 L/L (mean 0.38, S.D 0.03) RBC 2.53 $x10^{12}$/L (mean 6.20, S.D 0.72) and Hb 26g/L (mean 125.82, S.D 11.91).

Statistical analysis including comparison of cohorts via a one-way ANOVA was performed using SPSS® (SPSS.com, Chicago, Illinois, 60630, USA) Version 15 for Windows. Statistical significance was assessed at a level of $p<0.05$. Factors thought to significantly alter blood values included age (sub-adult compared to adults), origin (captive compared to wild), presence of *Treponema* infection and sex (males compared to females). *Treponema* infection in male Gilbert's potoroos presents as a moderate-severe balanoposthitis with an associated green tenacious discharge. *Treponema* infection was diagnosed via a number of techniques. In males, the penis was extruded and a comment made on the presence of any discharge and its characteristics. A dry wire swab (Mini-tip swabs, COPAN, USA) was used to swab the prepuce for the detection of *Treponema* infection via PCR assay. This swab was stored frozen at -20^{o}C degrees prior to transport via overnight courier to DAFWA. The prepuce was then swabbed with a moistened Amie's transport medium swab (Transwab®, Medical Wire & Equipment, England) which was first used to make a wet preparation smear for the detection of spirochaetes under dark field microscopy, then forwarded to the DAFWA for microbiological analysis and culture. In females the vagina was swabbed and a comment made on the presence of any discharge and its characteristics, and a dry wire swab (Mini-tip swabs®, COPAN, USA) was used for the detection of *Treponema* infection via PCR assay. The vagina was then swabbed with a moistened Amie's

transport medium swab (Transwab®, Medical Wire & Equipment, England) which was used to make a wet preparation smear for the detection of spirochaetes under dark field microscopy prior to being couriered overnight to DAFWA for microbiological analysis and culture. Definitive diagnosis of *Treponema* infection was associated with the presence of discharge, PCR positivity and positive dark-field microscopy for spirochaetes. Given the severity of associated clinical signs it was decided to examine hematological and biochemical differences between infected and non-infected individuals.

Results

The results of measured haematological and biochemical analytes are found in Table 1.

Despite careful collection, often in adverse weather conditions, some blood samples were slightly haemolysed. Haemolysis can alter the results of serum analytes.[1] However the values obtained from the haemolysed samples seemed comparable to unaffected samples and so were included in the results.

Blood smear examination

Examination of the blood film revealed the erythrocytes to be non-nucleated eosinophilic bioconcave discs with mild to moderate central pallor. Erythrocytes stained pale pink in Wright-stained preparations. Occasional Howell-Jolly bodies and mild anisocytosis was also evident in healthy individuals. Neutrophils had between 3-6 nucleur lobes with chromatin clumping and a colourless cytoplasm. Lymphocytes were typically small to medium-sized cells, although larger than the erythrocytes and had a round nucleus, chromatin clumping and basophilic cytoplasm. Monocytes had an irregularly shaped nucleus and a basophilic cytoplasm. Eosinophils when present had between 2-4 lobes with chromatin clumping and had diffuse eosinophilic granules present throughout the cytoplasm. Basophils had dark basophilic granules diffusely present throughout the cytoplasm. No alterations in leukocyte morphology were evident when comparing blood films of those with *Treponema* infection compared to those lacking *Treponema* infection.

Red cell inclusions were seen in three captive potoroos GP M46, F57 and F93 in June 2006, November 2006 and March 2007. There was a high degree of parasitaemia with at least two red cells infected per high power field. Morphologically the inclusions were described as pleomorphic, round, or irregularly shaped intra-erythrocytic parasites, approximately 1- 2µm in length. These inclusions were thought to be *Theileria* (Figure 2). They were distinguished by their size, the spherical ring surrounding the bodies, their presence in lymphocytes and the smaller amount of nuclear material compared to the piroplasm, *Babesia.* No evidence of anaemia, red cell morphology changes or inflammation as evidenced by a leukocytosis in peripheral blood was seen.

In June 2006, wild GP M68 and GP M116 had a similar high level of parasitaemia with *Theileria.* In November 2006 high parasitaemia with *Theileria* was again noted with a corresponding microfilaria infection in both individuals (Figure 3). This haemoparasite was identified to be *Breinlia* spp. due to its long unsheathed tapering tail.[5] Again there was no evidence of anemia, red cell morphology changes or inflammation as evidenced by a leukocytosis in peripheral blood in either individual.

Haematology and biochemical evaluation

Tests of normality conducted in SPSS® (Chicago, Illinois, 60630, USA) included the Kolmogorov-Smirnov statistic, a histogram, detrended normal Q-Q plot and a boxplot. Apart from monocytes (%), basophils (%), basophils ($x10^9$) and fibrinogen all haematological analytes were found to be normally distributed. The basophil and monocyte distribution was positively skewed with the majority of values clustered to the left of the histogram reflecting low values. Fibrinogen was similarly positively skewed to the left reflecting a majority of low values, however the kurtosis value reflected a lack of values in the centre of the distribution leading to asymmetry.

Serum biochemistry results of normality indicated a normal distribution of analytes other than CK, AST, Creatinine and Ca. For all of these analytes there was a low number of values in the centre of the distribution with a positive skew to the left indicating a trend towards low values. These four analytes also had one high outlier further skewing the distribution to the left minimising kurtosis and resulting in asymmetry.

Age, origin, sex and *Treponema* infection in relation to haematological and biochemical parameters were then examined. Age effects were evident in sub-adults compared to adults. Sub-adults returned significantly greater values for PCV (p=0.000), WBC (p=0.000), lymphocytes (p=0.000), basophils (p=0.017) total protein (p=0.033), fibrinogen (p=0.045), ALP (p= 0.000), glucose (p=0.05) and calcium (p=0.002) (Table 4).

Origin effects were evident in captive compared to wild individuals. Captive individuals had significantly greater values for Hb (p=0.000), PCV (p=0.000), RBC (p=0.000), ALP (p=0.03), cholesterol (p=0.000), and albumin (p=0.000). Wild individuals had significantly greater values for CK (p=0.001), AST (p=0.03), total bilirubin (p=0.004), urea (p=0.000), creatinine (p=0.000), globulin (p=0.002) and phosphorus (p=0.000) (Table 5).

Sex effects were minimal, males had significantly greater values for fibrinogen (p=0.029), creatinine (p=0.042) and cholesterol only (p=0.009). When comparing the *Treponema* infected population to the non-*Treponema* infected population again only minimal differences were seen in the following analytes. The *Treponema* infected individuals had lower cholesterol (p=0.041) and ALP (p=0.000) with higher serum protein (p=0.032) results. None of which were thought to be clinically significant and they all fell within the 95% confidence interval of the mean for the total population. No serological response was detected to any of the human based *Treponema* tests.

Discussion

Blood smear examination

The erythrocyte and leukocyte morphology was similar to that described by Clark[5] and Moore and Gillespie[17] in the Gilbert's and long-nosed potoroo respectively. The piroplasm, *Theileria* reported previously in Gilbert's potoroos[13] was found in three captive potoroos over three sequential trapping sessions spanning ten months. Two wild males were also found to have a high parasitemia, with at least two red cells infected per high power field. One of these individuals remained persistently infected for the following five months. This individual was not re-trapped in March 2007, as such no further disease screening for haemoparasites was undertaken.

In both the wild and captive population, the presence of *Theileria* was not associated with a typically regenerative anaemia, characterized by increased polychromasia of erythrocytes, reticulocytosis, macrocytosis, metarubricytosis, increased numbers of Howell-Jolly bodies, basophilic stippling[5] or inflammation as evidenced by elevated numbers of white blood cells in peripheral blood. The low numbers of Howell-Jolly bodies combined with the knowledge that Howell-Jolly bodies have been reported in clinically healthy individuals from numerous marsupial species[5] supported the findings of Lee[13] which did not indicate pathological consequences from the presence of *Theileria* in the Gilbert's potoroo. The observed high parasitaemia possibly reflected an acquired tolerance to *Theileria* in the Gilbert's potoroo.[13] This mechanism seems similar to the minimal host effect from *Theileria ornithorhynchi* found commonly in the platypus[15] suggesting that certain species of piroplasms, and marsupials and monotremes may have evolved together with minimal associated pathological effect.[13]

One wild male individual in sub-optimal body condition had evidence of a low level of infection with a single microfilariae found on peripheral blood smear. This haemoparasite was identified to be *Breinlia* spp. No corresponding elevation in white blood cells in the peripheral blood was seen. Given the prior reported presence of *Breinlia* spp. and *Breinlia macropi* in Australian marsupials, including the long-nosed and Gilbert's potoroo, the pathological consequences of this

haemoparasite warrant discussion. *Breinlia macropi* was isolated from the peritoneal and pleural cavities of an aged female Gilbert's potoroo found dead assumed to have been recently regurgitated by a python. While the presence of *Breinlia macropi* microfilaria in a swamp wallaby caused eosinophilia in peripheral blood, and an associated microfilaraemia.[5] Reports in other marsupials are based on incidental findings of sub-clinical infection with this parasite. Given the low levels of microfilaria seen, the lack of associated eosinophilia in peripheral blood and the fact that the regurgitated potoroo seemed to be suffering from many concomitant diseases, it is difficult to assign too much significance to the presence of microfliaria in this individual. However, blood smears should continue to be evaluated for the presence of microfilaria to allow a more thorough assessment of its significance to be made. Concentration techniques to enhance detection could also be considered.

It is recommended that haemoparasite screening via blood smear should continue to be undertaken as part of health screening any potoroo to be translocated or any potoroo undergoing health evaluation for diagnostic purposes.

Haematology and biochemical examination

Numerous blood reference ranges were examined for marsupials. [3,5,9,10,17,19,20,24,25,27,28,29] Many of these studies reported haematological findings with no biochemistry being available or alternatively, the methods of biochemical analysis were not reported making comparison of analytes difficult.

The mean WBC count for the Gilbert's potoroo was 3.118 x 10^9/L ± 1.03. This value is considerably lower than that of the long-nosed potoroo, (*Potorous tridactylus)* 8.06 x 10^9/L ± 2.59[13] and the rufous bettong (*Aepyprymnus rufescens)* 6.49 x 10^9/L ± 0.63 (Spencer *et al.* unpublished data). These potoroid species were thought to be the most similar in terms of digestive system to the Gilbert's potoroo, and as such serve as a useful comparison.

Like most marsupials, the predominant circulating leukocyte in the Gilbert's potoroo was the lymphocyte.[5] The absolute mean number of lymphocytes (1.89 x10^9/L) was comparable to other marsupial species including the mountain brushtail possum *Trichosurus caninus* (1.6 x 10^9/L)[28] yet considerably different from wild-allied rock wallabies *Petrogale assimilis* (5.22 x10^9/L);[23] the long-nosed potoroo, [16] and the rufous bettong (Spencer *et al.* unpublished data). However it should be highlighted that only relative values were reported for the analogous potoroid species, no absolute values were reported.

The neutrophil:lymphocyte (N:L) ratio is thought to be a reliable method to detect glucocorticoid mediated 'stress' in captive animals.[19] However, only absolute concentrations of neutrophils and lympocytes were used in this study as the N:L ratio may change in healthy individuals, without causing the absolute values to be outside of the reference interval. In a classic glucocorticoid mediated stress response an absolute neutrophilia and lymphopenia is observed leading to an increased N:L ratio. Less regularly a mild eosinopenia may also be found.[5] Using the mean leukocyte values, the calculated mean N:L ratio for the total population of the Gilbert's potoroo was 0.49. Given no statistical significance in leukocyte count was found between wild and captive individuals it was not thought necessary to compare these two cohorts. Marsupials tend to have N:L ratios of less than 1.[19,29] Agile wallabies (*Macropus agilis*) have a N:L ratio of 0.5[24] and quokkas (*Setonix brachyurus)* have a reported N:L ratio of 0.82.[14] The value found in the Gilbert's potoroo although low, still appears to be within close range to other macropodoids. Absolute N:L ratios were not available for the long-nosed potoroo or rufous bettong.

Alterations in the N:L ratio have been observed with variations in age, method of restraint and methods of blood collection.[26] Sub-adult Gilbert's potoroos had a lower neutrophil:lymphocyte ratio (0.29) compared to adults (0.66) as sub-adults had a statistically higher lymphocyte count (P=0.000). Likewise in allied rock wallabies the neutrophil concentration increased with age and resulted in an increase in the N:L ratio from 0.33 at 150 days to 1.0 at maturity.[24] Although the

sub-adult cohort in this study extended to animals between 225 to 250 days post-birth the N:L ratio still remained relatively low at maturity (0.66) compared to other marsupials.

The reason for the trend of an increased N:L ratio with age seems to be a physiological response with a predominance of immature myeloid cells being observed in the blood of marsupial pouch young, with a gradual increase in the number of neutrophils seen over time. This response has also been reported in the eastern quoll (*Dasyurus viverrinus*), common brushtail possum (*Trichosurus vulpecula*) and southern brown bandicoot (*Isoodon obesulus*).[6] Although the capture technique of captive compared to wild individuals differered substantially leukocyte changes were not statistically significant. This is interesting given that free-living koalas (*Phascolarctos cinereus*) had a persistent neutrophilia up to six hours post-capture[9] and free-living platypus (*Ornithorhyncus anatinus*) had a persistent neutrophilia up to 12 hours post capture.[28] However, neutrophilia may also be due to an inflammatory process, (infectious or non-infectious) or may be indicative of sub-clinical disease.

Although leukocyte changes were not significant, higher muscle enzyme activity of CK and AST were evident in the wild compared to the captive population. Elevation of these enzymes is indicative of muscle injury often associated with stressful restraint or capture.[26] Furthermore the corresponding lack of liver enzyme elevation in these wild individuals indicated the AST increase was more likely to be associated with the muscle iso-enzyme, rather than liver function. Wild individuals could potentially be trapped for up to 12 hours prior to physical examination and anaesthesia, while captive individuals were captured no longer than one hour prior to physical examination and anesthesia. Although every attempt was made to minimize the stress of capture of wild individuals through wrapping traps in hessian bags to minimize exposure to the elements and predators, the potential for muscle enzyme elevation with overnight trapping must be considered when comparing these two cohorts.

Sub-adult Gilbert's potoroos had a higher packed cell volume, and total protein level compared to adults. Age related changes characterized by an increasing Hb, RBC and haematocrit were reported in the quokka,[29] koala, allied rock-wallaby and common brushtail possum.[5] The only marsupial species reported to decrease its haematocrit with age is the female mountain brushtail possum.[4]

Wild potoroos had a significantly decreased Hb concentration, PCV and numbers of RBC. Anaemia in macropods has been associated with haemorrhage from nematode infestation[2] and other endoparasites.[23] A dominance of strongyloid nematodes was found in wild Gilbert's potoroos over the course of this study however the lack of associated clinical signs and absence of anaemia led these endoparasites to be regarded as commensal and of little clinical significance. The increased Hb concentration, PCV, and RBC count relative to the reference interval indicated that the captive population was polycythaemic. This physiological response occurs for two reasons; a decrease in plasma volume (relative polycythaemia) or an increase in cells (absolute polycythaemia). The concomitant increase in plasma albumin in the captive population could be explained by a decreased plasma volume and loss of water from the vasculature owing to reduced fluid intake, vomiting, diarrhoea or diuresis.[26] However, all captive individuals constantly had water available and very infrequently had gastro-intestinal symptoms. It seems more likely that the erythron changes represent a re-distribution or erythrocytes following catecholamine-mediated splenic contraction[4] in response to capture. In the koala *(Phascolarctos cinereus*), a similar phenomenon is observed with the haemoglobin concentration, erythrocyte concentration and PCV being highest at the time of capture, then decreasing stepwise at 6 hours, 24 hours and 7 days post capture.[9] This is interesting as the captive Gilbert's potoroos are handled fortnightly and these elevations in the erythron could be indicative of ongoing catecholamine-mediated splenic contraction. However the corresponding lack of leukocyte changes seems to dispel this theorem as a corresponding neutrophilia and lymphocytosis would also be expected.

Other differences in sub-adult compared to adult Gilbert's potoroos included significantly greater values for total protein (p=0.033), fibrinogen (p=0.045), ALP (p= 0.000), glucose (p=0.05) and calcium (0.002). Fibrinogen is an acute phase reactant protein, stimulated by pro-inflammatory cytokines and is a useful marker of inflammation in a variety of species.[8] Reference ranges in marsupials are typically from 1-4 g/L.[5] However the time required for the protein concentration to become significantly increased and the duration of the increased concentration, is based upon both the severity of inflammation and the species affected. Sub-adults had a higher fibrinogen concentration. There were no signs of clinical disease or inflammation, other than physiological changes associated with immaturity including an increased white cell count, lymphocyte and basophil count. However when associated with an elevated PCV and total protein the possibility of mild dehydration in the sub-adult cohort, leading to a relative increase in blood protein fractions, including fibrinogen should be considered. Correspondingly the increase in serum calcium relative to the adult cohort may be reflective of increased total protein levels, driving more protein-bound calcium into the circulation.

Increased serum ALP activity associated with increased osteoblastic activity occurs in all species.[25] The elevation in ALP in sub-adults is probably explained by the ALP isozyme in bone which may cause an elevation of serum ALP in young growing animals.[21,22]

The blood glucose value in sub-adults was slightly higher than the mean value for adults. This was most likely associated with stress or excitement from handling, fitting with the observed elevated PCV and leukoyctosis. Yet, the typical leukoid glucocorticoid mediated 'stress' response observed by neutrophilia and lymphopaenia was not observed. Overall this indicates a lack of consistency between reported haematological markers of stress and the results of blood samples from Gilbert's potoroos.

In relation to serum biochemistry, wild Gilberts potoroos had a greater total bilirubin (p=0.004), urea (p=0.000), creatinine (p=0.000), globulin (p=0.002) and phosphorus (p=0.000) compared to captive individuals. The relative increased urea, creatinine, globulins and phosphorus levels may point to a reduction in the glomerular filtration rate, resulting from a pre-renal, renal or post-renal cause. A pre-renal cause could include dehydration. Unlike their captive counterparts the wild population would not have ongoing access to water and would maintain their hydration mostly through the ingestion of tubers and truffles to obtain their water requirements. In a separate study urinalysis values and in particular urine specific gravity was analysed using a one way ANOVA and a mean of 1.021 with a range of 1.004 and 1.039 was recorded with a standard error of 0.009 (n=33). These values are similiar to those found in companion animals.[25] A healthy hydrated dog should have a USG between 1.015 and 1.045.[12] Extrapolating from these findings we could then comment that the majority of Gilbert's potoroos tend to be well hydrated. Furthermore no statistical difference between captive and wild individuals was found (p = 0.19) when assessing urine specific gravity. Other renal or post-renal causes for the observed differences in the wild and captive population may include, balanoposthitis caused by *Treponema* or renal oxalosis. It is unlikely the balanoposthitis is involved as when blood values of *Treponema* infected compared to non-*Treponema* infected individuals were compared no significant differences in the above analytes were found. Furthermore balanoposthitis caused by *Treponema* is present in both the captive and wild population. A more likely explanation of the increased analytes in the wild population may be linked to the present lack of captive individuals with the presumably familial renal oxalosis. Renal oxalosis has been screened through urinary glycolate testing via DEC staff and the captive population's diet has been modified to minimise oxalate consumption (D.Forshaw, personal communication May 2007). Renal oxalosis refers to the microscopic deposition of oxalate in the tubules causing tubular epithelial necrosis and dysfunction.[13] Severe renal oxalosis was the cause of death of five animals in the captive population. Four of these deaths occurred in the one family group with the high incidence in related individuals suggestive of an inherited condition (Horwitz and Forshaw 2001, unpublished data). Excess dietary oxalate intake was eliminated as a possible cause of the renal oxalosis. At present, a probable inherited disorder of oxalate metabolism involving the enzyme pathways around the intermediary substrate glyoxylate (degraded to oxolate and glycolate) and enzyme

mis-targetting is the most likely explanation (Horwitz and Forshaw 2001, unpublished data). However, the possibility of other unprecedented mechanisms cannot be dismissed. It is presumed to still exist in the wild population and the values from these analytes indicate that further urinary glycolate testing should be undertaken.

The age distribution in the captive compared to wild cohort was also compared. No statistical significance in renal parameters was found between sub-adults and adults, as such no significant relationship between age and chronic renal disease was apparent. Aged male GP 55, who was excluded from the study was the only exception.

Elevated bilirubin was also evident in the wild compared to the captive cohort. Bilirubin concentration can be increased through increased production of haemoglobin owing to increased erythrocyte destruction, decreased uptake or conjugation of bilirubin by hepatocytes and disruption of bile flow.[25] Certainly the wild cohort had a statistically significant elevation in AST compared to the captive population. However, given an elevated CK was also present, muscle iso-enzyme induction was assumed and hepatic hyperbilirubinaemia was not suspected. Increased erythrocyte destruction was not present evidenced by the erython values. Retention or fasting hyperbilirubinaemia occurring through anorexia or starvation is the most likely cause of the observed values as wild individuals could potentially have been trapped 12 hours prior to anesthesia for blood collection and, given they are nocturnal, may not have eaten for the preceding 12 hours. Although once trapped the potoroos had access to the bait material, this was not always eaten. Captive potoroos were not fasted, and still received their afternoon meal, as such a fasting hyperbilirubinaemia was not seen.

The captive population had a greater level of cholesterol compared to the wild population. However, no samples were grossly lipaemic. Although the mean of the captive cohort was outside the upper 95% confidence interval for the mean cholesterol value in Gilbert's potoroos, this increase is thought to be dietary related rather than pathological. All captive Gilbert's potoroos were well conditioned to obese (body condition score 3.5 to 5). The potoroos were scored based on the amount of fat overlying their thoraco-lumbar spine and tail base. Body condition was graded from one to five, one being emaciated and five being obese. Wild potoroos had a condition score of 2 and below. The diet of captive potoroos is based on the wild diet, where fungal material (truffles) was found to make up more than 90% of the diet.[17] However, owing to seasonal variation the captive diet now consists of 10% (by weight) of hypogeal fungi[5] as well as mealworms, nuts, cereal, fruit and vegetables. The fat content, especially from the mealworms and nuts, possibly accounts for the higher observed cholesterol level.

The minimal haematology and biochemistry differences between the sexes is not surprising as numerous marsupial studies have reported no differences attributable to sex in the total leukocyte and differential leukocyte concentrations in common brushtail possums,[19] mountain brushtail possums[27] or allied rock-wallabies.[24]

The minimal haematological and biochemical differences between *Treponema* infected and non-infected individuals is also not surprising as the organism appears to be locally invasive with no serological response detected through using the *Treponema pallidum* particle agglutination (TPPA), Enzyme immunoassay (EIA), and the non-specific Rapid plasmid reagin (RPR) test . A venereal spirochaetosis has been identified in rabbits caused by *Treponema paraluis-cuniculi* which results in vulval and preputial lesions beginning as areas of hyperemia and oedema with the development of papules and vesicles followed by ulceration, crustiness and hyperkeratosis.[6] This is very similar to the clinical presentation seen in Gilbert's potoroos. Like rabbits, potoroos did not seem to develop leukocytosis with *Treponema* infection, yet, unlike rabbits they were non reactive to the RPR test. This is the test currently used as a diagnostic aid for treatment success in the rabbit.[7]

The data from this study was used to create the first haematology and biochemistry reference intervals for Australia's most critically endangered mammal, the Gilbert's potoroo. 80 samples

were collected from a population of only 35 individuals reflective of a population census rather than a study reliant on statistical extrapolation. Standardised capture, collection and processing protocols were used to optimize data quality. These reference ranges will assist in the ongoing health management of the Gilbert's potoroo and provide a useful comparison for other potoroid species both in the wild and in captivity.

ACKNOWLEDGEMENTS:
We acknowledge the work and advice of Stephanie Hill, Tim Button, Val Hack, Susanne Schreck (Department of Environment and Conservation, Albany) and the Gilbert's Potoroo Recovery Team. Thank you also to Dr David Forshaw of the West Australian Department of Agriculture and Food for ongoing advice. The staff of the Perth Zoo Veterinary Department and Murdoch University Laboratories (Murdoch, Western Australia, Australia) are also to be acknowledged for providing results of clinical pathology. Dr Mary McConnell from Vetpath laboratories in Perth, Western Australia is also to be thanked for her input into the formulation of blood reference ranges in populations with low numbers.

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Table 1. Haematological and biochemical values for Gilbert's potoroo.

Analytes n=35		Mean	Standard error	Upper 95% C.I for mean	Lower 95% C.I for mean	Standard Deviation	Minimum value	Maximum value
Hemoglobin (g/L)	Hb	125.82	3.59	133.82	117.82	11.91	94	157
Hematocrit (L/L)	HCT	0.38	0.11	0.41	0.36	0.04	0.29	0.49
Packed cell volume (L/L)	PCV	0.36	0.11	0.39	0.34	0.037	0.28	0.46
Red blood cell count	RBC	6.20	0.22	6.69	5.72	0.72	2.53	8.24
Mean cell Hb concentration	MCHC	330.27	3.20	337.41	323.13	10.63	303	354
Mean corpuscular Hb	MCH	20.34	0.32	21.05	19.62	1.06	18.6	21.8
Mean corpuscular volume	MCV	61.6	0.96	63.73	59.47	3.175	56.10	61.72
White blood cell count	WBC	3.12	0.31	3.81	2.43	1.03	0.60	9.40
Neutrophils (%)	Neut	33.27	4.26	41.76	22.79	14.12	2	72
Neutrophils (x 10^9/L)	Neut	0.93	0.11	1.172	0.69	0.35	0.16	2.23
Lymphocytes (%)	Lymph	58.36	4.96	69.41	47.32	16.44	26	90
Lymphocytes (x 10^9/L)	Lymph	1.89	0.31	2.58	1.22	1.01	0.23	7.13
Monocytes (%)	Mono	1.68	0.32	0.24	0.97	1.05	1	10
Monocytes (x 10^9/L)	Mono	0.49	0.01	0.07	0.03	0.03	0.01	0.20
Eosinophils (%)	Eosin	2.73	0.43	3.68	1.77	1.42	0	15
Eosinophils (x 10^9/L)	Eosin	0.08	0.02	0.13	0.04	0.06	0.03	0.59
Basophils (%)	Baso	1.36	0.20	1.82	0.91	0.67	1	3
Basophils (x 10^9/L)	Baso	0.43	0.01	0.06	0.023	0.03	0.02	0.11
Platelet Count (x 10^9/L)		345.45	39.55	433.59	257.32	131.19	71	578
Total Protein (g/L)	TP	61.09	0.59	62.42	59.77	1.97	56	75
Fibrinogen (g/L)		1.45	0.21	1.91	0.98	0.69	1	3
Creatine kinase (U/L)	CK	1074.14	260.39	1636.68	511.6	924.29	157	8978
Aspartate transferase (U/L)	AST	84.64	11.59	109.68	59.6	43.36	44	161
Alanine aminotransferase (U/L)	ALT	76.93	7.19	92.48	61.38	26.93	33	298
Alkaline phosphatase (U/L)	ALP	318.64	39.19	403.32	233.96	146.66	88	1327
Total bilirubin (mmol/L)	BILI	4.69	0.91	6.66	2.72	3.41	0	12
Urea (mmol/L)	BUN	5.95	0.41	6.81	5.08	1.50	2.10	28.60
Creatinine (mmol/L)	CREAT	49.52	3.48	57.05	41.99	13.04	33	334
Glucose (mmol/L)	GLU	9.21	0.61	10.54	7.87	2.30	5.80	20.10
Cholesterol (mmol/L)	CHOL	3.75	0.22	4.23	3.272	0.83	26	6.50
S Protein (g/L)	SP	56.61	1.67	60.22	53.01	6.24	45.30	73.10
Albumin (g/L)	ALB	33.24	0.59	34.51	31.96	2.21	28	43
Globulin (g/L)	GLOB	23.36	1.74	27.13	19.60	6.51	13.70	36.10
A/G ratio	A/G	1.54	0.14	1.84	1.25	0.51	0.94	2.75
Calcium (mmol/L)	Ca	2.03	0.06	2.159	1.89	0.23	1.30	2.62
Phosphorus (mmol/L)	P	2.84	0.25	3.38	2.30	0.94	1	4.5

Figure 2. Peripheral blood smear of a Gilbert's potoroo showing the presence of a. a Howell-Jolly body and b. intra-erythrocytic *Theileria* (b). The piroplasms were round, ovoid or pear shaped with 1-2 parasites present per cell. Haemoxylin and Eosin 100x.

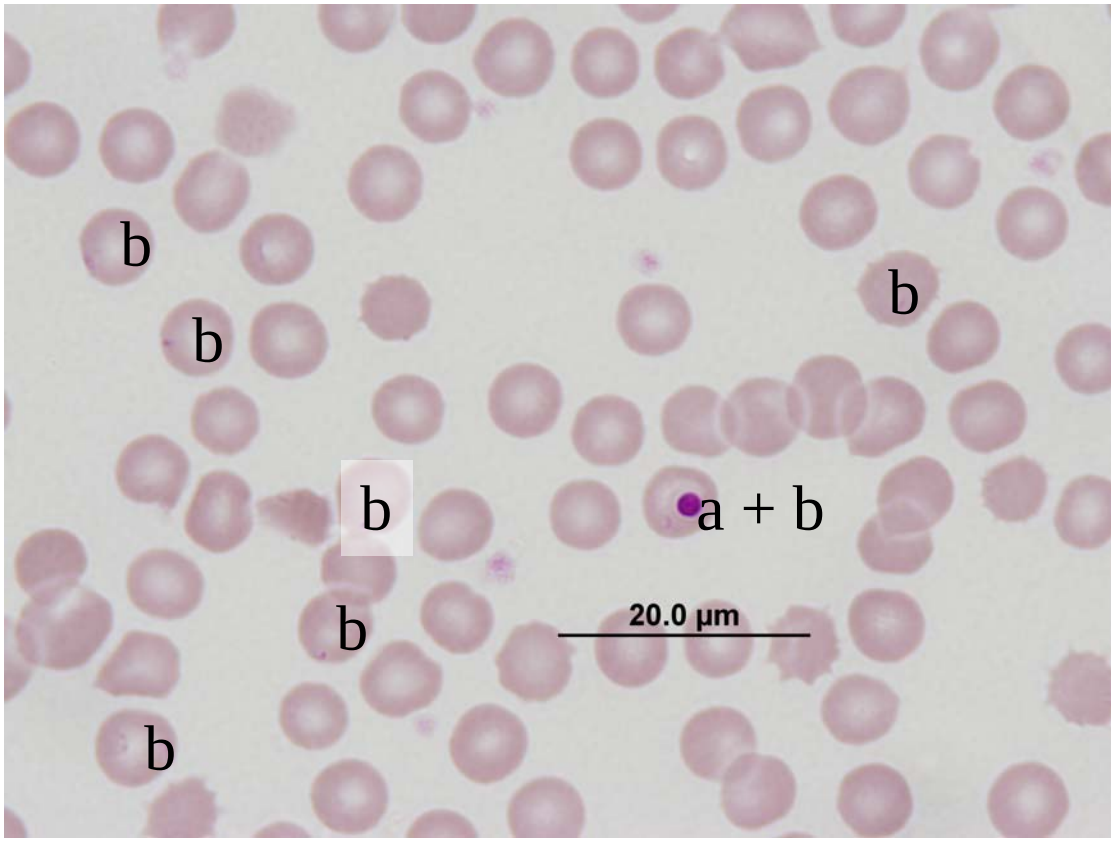

Figure 3. Microfilaroid in a peripheral blood smear of a Gilbert's potoroo. Haemoxylin and Eosin 40x.

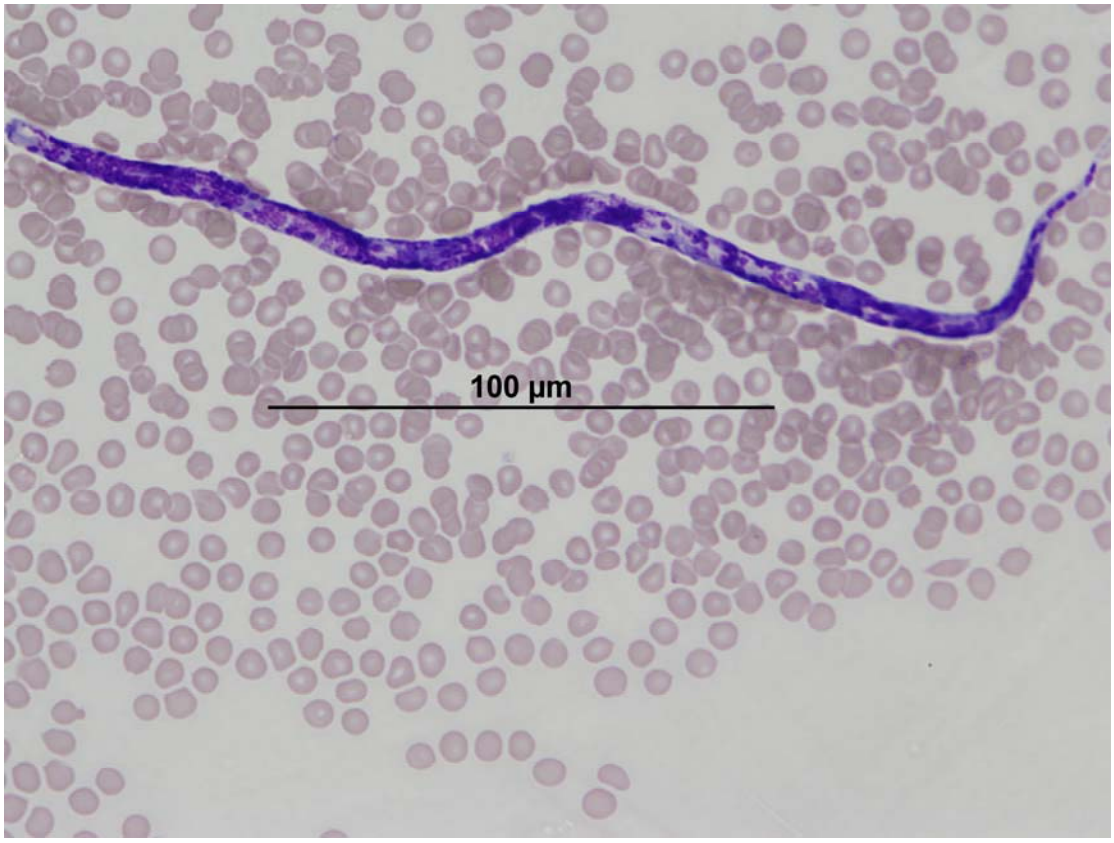

Table 4. Statistically significant effects of age on haematological and biochemical values for Gilbert's potoroo.

Analytes n=35	Sub-Adult (SA) (n=12) or Adult (A) (n=23)	Mean	Standard error	Upper 95% C.I for mean	Lower 95% C.I for mean	Standard deviation	Minimum value	Maximum value
Packed cell volume (L/L)	SA	0.37	0.01	0.45	0.29	0.30	0.33	0.46
	A	0.36	0.01	0.39	0.32	0.04	0.28	0.44
White blood cell count	SA	4.33	0.67	7.23	1.43	1.17	2.00	9.40
	A	2.66	0.18	3.08	2.24	0.51	0.60	5.10
Lymphocytes (%)	SA	75	2	83.61	66.39	3.46	45	90
	A	52.13	5.24	64.51	39.74	14.81	28	88
Lymphocytes (x 10^9/L)	SA	3.22	0.42	5.01	1.42	0.72	0.95	7.13
	A	1.41	0.19	0.90	1.86	0.94	0.23	4.08
Basophils (%)	SA	1.67	0.33	3.10	0.23	0.57	1	2
	A	1.25	0.25	1.84	0.66	0.71	1	3
Basophils (x 10^9/L)	SA	0.07	0.02	0.174	0.00	0.04	0.03	0.11
	A	0.03	0.01	0.04	0.02	0.01	0.02	0.07
Total Protein (g/L)	SA	61.33	0.88	65.13	57.54	1.53	59.00	65
	A	61	0.78	62.84	59.16	2.20	50.00	75
Fibrinogen (g/L)	SA	1.67	0.67	4.54	1.2	1.15	1.00	3.00
	A	1.36	0.19	1.8	0.92	0.53	0.90	3.00
Alkaline phosphatase (U/L)	SA	467.25	90.19	754.28	180.22	180.38	54.00	152
	A	259.2	25.46	316.8	201.6	80.52	88.00	481
Glucose (mmol/L)	SA	9.87	1.40	14.34	5.40	2.81	6.40	20.10
	A	8.94	0.69	10.50	7.37	2.18	6.00	17.50
Calcium (mmol/L)	SA	2.11	0.18	2.69	1.53	0.37	1.79	2.62
	A	1.99	0.05	2.11	1.88	0.16	1.30	2.32

Table 5. Statistically significant effects of origin on haematological and biochemical values for Gilbert's potoroo.

Analytes n=35	Origin: Wild (n=26)or Captive (n=9)	Mean	Standard error	Upper 95% C.I for mean	Upper 95% C.I for mean	Standard deviation	Minimum value	Maximum value
Hemoglobin (g/L)	Wild	124	5.08	136.43	111.57	13.44	94.90	141
	Captive	129	4.74	144.10	113.9	9.48	115	157
Packed cell volume (L/L)	Wild	0.35	0.01	0.38	0.31	0.04	0.28	0.42
	Captive	0.39	0.01	0.41	0.37	0.01	0.33	0.44
Red blood cells	Wild	5.96	0.27	6.62	5.31	0.71	2.53	7.34
	Captive	6.63	0.30	7.58	5.67	0.60	5.37	7.85
Creatine kinase (U/L)	Wild	1327	332.01	2078.06	575.94	1049.91	395	8978
	Captive	442	122.97	833.37	50.63	245.95	430	3694
Aspartate transferase (U/L)	Wild	88.5	16.19	125.12	51.88	51.19	51	417
	Captive	75	5.54	91.99	58.01	10.67	55	273
Alkaline phosphatase (U/L)	Wild	309.9	53.35	430.59	189.21	168.71	88	878
	Captive	340.5	41.6	472.92	203.08	83.22	137	1327
Total Bilirubin (mmol/L)	Wild	5.6	1.01	7.89	3.31	3.20	0.90	11.90
	Captive	2.43	1.57	7.44	0.00	3.154	0.10	8.00
Urea (mmol/L)	Wild	6.64	0.35	7.45	5.87	1.09	3.5	11.40
	Captive	4.15	0.19	4.76	3.33	0.39	2.1	11.80
Creatinine (mmol/L)	Wild	49.73	4.91	60.83	36.83	15.52	33	90
	Captive	49	1.87	54.95	43.05	3.74	35	139
Cholesterol (mmol/L)	Wild	3.34	0.02	3.94	2.97	0.67	2.70	4.40
	Captive	4.49	0.37	5.69	3.29	0.75	2.70	6.52
Albumin (g/L)	Wild	32.26	0.50	33.39	31.22	1.59	27.80	43
	Captive	35.67	0.79	38.19	33.16	1.58	31.20	40.10
Globulin (g/L)	Wild	25.85	1.85	30.04	21.65	5.86	15.30	36.10
	Captive	17.15	1.47	21.84	12.46	2.94	13.70	27.70
Phosphorus (mmol/L)	Wild	3.19	0.26	3.78	2.59	0.84	1.50	4.50
	Captive	1.97	0.27	2.85	1.1	0.55	1	4.20

APPENDIX 14: Urinalysis values in the Gilbert's potoroo.

Unpublished report for internal use by the Gilbert's Potoroo Recovery Team February 1 2008.

Introduction

A collaborative health and disease study of the Gilbert's potoroo was undertaken involving the Department of Environment and Conservation, the Department of Food and Agriculture and the Perth Zoo. The research aimed to determine the prevalence of specific diseases in the wild and captive populations and to correlate the effects of identified diseases on population dynamics including reproductive success and survivorship. Diseases screened for included *Cryptococococcosis*, endoparasitism, ectoparasitism, haemoparasitism, *Toxoplasmosis*, and *Treponema* infection. Physical examination, and blood and urine collection was also undertaken to evaluate the general health of the Gilbert's potoroos. In total 33 urine samples were obtained over a three year period from 21 Gilbert's potoroos. Urine was collected to establish reference urine values, and to identify effects of disease on urine values.

The disease thought to be of most significance to urine findings was *Treponema* infection which has been associated with a balanoposthitis in both the wild and captive population of the Gilbert's potoroo. Clinically this is evident as crusty green preputial discharge often with severe associated preputial and cloacal inflammation and ulceration. Bacteriological examination has revealed a number of potential pathogens amongst the mixed bacteria isolated. However, the effects of this infection on urine values has yet to be assessed. Infection of the urogenital tract is typically associated with increased numbers of white blood cells and red blood cells. Inflammation is differentiated from haemorrhage on the basis of the ratio of leukocytes to erythrocytes (Thrall 2004). If the two cell types are present in a similar proportion in the peripheral blood the process is more likely to be haemorrhage, while if leukocytes are more numerous than in the peripheral blood, inflammation is more likely. Proteinuria can also be expected in conjunction with haemorrhage, inflammation or renal tubular degeneration (Thrall 2004).
The method of urine collection is also important when assessing the likelihood of infection as the site of infection is often difficult to ascertain if standard free catch urine samples are obtained. A free-catch sample with increased white and red blood cells may be indicative of infection anywhere along the urogenital tract, where as a sterile cystocentesis sample or catheterized sample, pinpoints the bladder as the source of infection (Davidson *et al.* 1988).

Another disease thought to be of significance to this study was renal oxalosis. Renal oxalosis historically caused the death of five individuals in the captive colony (Horwitz and Forshaw 2001, unpublished data). Renal oxalosis refers to the microscopic deposition of oxalate in the renal tubules causing tubular epithelial necrosis and dysfunction (Moffatt 1977). Four of these deaths occurred in the one family group with the high incidence in related individuals suggestive of an inherited condition (Horwitz and Forshaw 2001, unpublished data). No captive individuals currently have any evidence of renal oxalosis, however it continues to been screened for through urinary glycolate testing via Department of Environment and Conservation (DEC) staff and the captive population's diet has been modified to minimize oxalate consumption (D.Forshaw, personal communication, May 2007). At present, a probable inherited disorder of oxalate metabolism involving the enzyme pathways around the intermediary substrate glyoxylate (which is degraded to oxolate and glycolate) and enzyme mis-targetting is the most likely explanation (Horwitz and Forshaw 2001, unpublished data). It is presumed to still exist in the wild population and the values from renal biochemistry data (Appendix 13) indicate that further urinary glycolate testing should be undertaken.

Calcium oxalate and calcium carbonate crystalluria is commonly found in other herbivores including horses and cattle. These crystals typically occur in neutral to acidic urine and derive from the high oxalate content of a herbivorous diet. Calcium oxalate crystalluria occurs less commonly in dogs and cats owing to their carnivorous diet. If persistent, it may indicate an increased risk for calcium oxalate urolith formation (Thrall 2004).

Other crystals commonly observed in companion animal urine include magnesium ammonium phosphate crystals or struvite crystals (Kahn and Line 2005). Struvite crystalluria in dogs is not pathological unless there is a concurrent bacterial urinary tract infection with a urease-producing microbe. However, some animals (e.g. cats) can form struvite uroliths without a bacterial urinary tract infection. In these animals, struvite crystalluria may be pathologic.

A paucity of reported urinalysis and sediment examination findings in marsupial and monotreme species exists. In echidnas and platypus urinary urea, uric acid, allantoin levels and pH were reported (Denton *et al.* 1963; Mitchell 1931) and in platypus one urine sample from a moribund individual was analysed (Whittington 1988). Reference intervals have only been reported in the koala presumably owing to the prevalence of chlamydial infections (Canfield *et al.* 1989). In Canfield's paper, urinalysis was performed on 20 urine samples collected at seven visits from 14 healthy captive koalas and from samples from five koalas with cystitis. However, when comparing these values to those of the Gilbert's potoroo the differing digestive strategies and diet should be remembered. Koalas are entirely herbivorous eating only the leaves of *Eucalyptus*, correspondingly they have a high gastric pH which is required to break down the fibrous diet and highly concentrated urine owing to the variable but low water content of *Eucalyptus* (Canfield *et al.* 1989). The diet of captive potoroos is based on the wild diet, where fungal material (i.e. truffles) make up more than 90% of the diet (Nguyen 2000). However due to seasonal variation of fungi, the captive diet now consists of 10% (by weight) of hypogeal fungi (Courtney and Friend 2004), as well as mealworms, cheese, yoghurt, fruit and vegetables. This diet contains a much higher water content, therefore less concentrated urine would be expected in captive individuals.

Over these three years six, three week trapping sessions were undertaken with the number of potoroos sampled at each session demonstrated below (Table 1). Many of the captive and wild individuals were re-trapped over the six trapping sessions. However, given there was a minimum of three months between sampling, the use of these repeated samples in the population data was thought to be justifiable. The number of urine samples collected generally differed from the number of individuals trapped at each trapping session as most potoroos urinated prior to restraint for physical examination and anaesthesia, resulting in fewer individuals from which urine could be collected. This event is not uncommon when trapping marsupials and is thought to be associated with the stress of capture. Another factor altering the population sampled was the presence of pouch young which were thought to be of 'critical size' in relation to vulnerability and survivability. If females were trapped with pouch young of a 'critical size' then general anaesthesia and urine collection was not undertaken. Although this may place some bias on the population studied, only four females belonged to this category, and it was though the relative risk of this procedure for the pouch young, far outweighed the value of assessing urine parameters for this cohort of Australia's most critically endangered mammal.

Figure 1. Individuals sampled at each trapping session

Bolded individuals refer to members of the captive colony

Potoroo ID	*Mar-05*	*Jun-05*	*Nov-05*	*Jun-06*	*Nov-06*	*Mar-07*	TOTAL
M28	X						1
M46						X	1
M49		X		X			2
M55		X	X	X	X		4
F61			X			X	2
F66		X					1
M68		X	X				2
F69			X				1
M77		X					1
M83			X	X	X	X	4
F89		X					1
M94			X	X		X	3
F98		X					1
F101		X					1
M104			X				1
F106			X				1
M107				X		X	2
M111				X			1
M116					X	X	2
F119						X	1
TOTAL		**9**	**8**	**6**	**3**	**7**	**33**
WILD		**7**	**8**	**5**	**3**	**5**	**28**
CAPTIVE		**2**	**0**	**1**	**0**	**2**	**5**

Materials and methods

Wild potoroos were trapped three times per year (March, June and November) by Department of Environment and Conservation staff to monitor the population in accordance with the Gilbert's Potoroo Recovery Plan ratified by the Gilbert's Potoroo Recovery Team. On Mount Gardner, Sheffield cage traps, wrapped in hessian to minimize exposure to the elements, were set the afternoon prior to trapping and baited with a pistachio-nut flavoured muesli ball. These traps were checked at first light. When a potoroo was found it was transferred to a black cotton bag and a total weight obtained with the use of spring scales. The bag was also scanned with a Trovan® portable scanner for the presence of a microchip to enable identification of the individual animal. All potoroos were assigned a number that correlated with their microchip number. Captive Gilbert's potoroos were captured using a shade cloth run and net on a fortnightly basis to enable regular weighing and physical examination.

Initial health assessment was carried out via visually checking for faecal soiling, urogenital discharge, assessment of body weight and body condition scoring. Complete health assessment was carried out under general anaesthesia.

To undertake the procedures required for disease testing the potoroo's nose was exteriorized from the black cotton bag and the animal was induced with Isoflurane (Veterinary Companies of Australia, Kings Park, NSW) delivered via face mask at 3.5% Isoflurane with an oxygen flow rate of 2.5L/minute. Five minutes post induction most potoroos were able to be maintained on 1.5% Isoflurane and 2L/minute of oxygen. A pulse oximeter was available from June 2005 enabling the electronic monitoring of heart rate and oxygen saturation. These parameters were obtained by placing the probe on the external pinna or between the toes of the anaesthetised potoroo. Anaesthetic and general physical exam data sheets were completed every time an individual was anaesthetised for disease testing. This enabled anaesthetic and physical examination records of abnormalities to be retained for subsequent trapping sessions.
A physical examination, cloacal and urogenital swabs and blood collection for a general health profile was undertaken. Following this the bladder was palpated and manually expressed. A mid-stream, free catch sample was obtained. This sample was then forwarded via overnight courier to Murdoch University for urinalysis, and sediment exam. Culture was indicated if large numbers of inflammatory cells were evident microscopically.

Glucose, Bilirubin, ketones, blood, pH, protein and urobilinogen were analysed using Multistix® Reagent strips (Bayer, Pymble, Australia). 3% salicylsulphonic acid precipitation was used to obtain the Sulphosalicylic acid concentration (Varley 1967) and a refractometer was used to obtain the Specific Gravity of the supernatant. An aliquot of urine was centrifuged at 1500 rpm for 5 minutes to produce the sediment which was then examined underneath a light microscope for inflammatory cells, bacteria, crystals, casts, mucous threads and sperm.

Data was recorded using an Excel spreadsheet and an assessment of urine parameters made in light of physical examination findings, recovery post procedure and the absence of any history of current or chronic illness. Those individuals deemed to be unhealthy were excluded from the study. Four individuals were deemed unhealthy.

- In March 2005, captive male 28 voided brown, odiferous urine with a USG of 1.0, pH 8.5, that was positive for protein (2+), WBC were reported as 'too numerous to count' as were the numbers of RBC/hpf. This individual was also infected with *Treponema* and bacteria were seen on sediment exam. The swab from the rectal opening within the cloaca revealed a mixed infection with *E.coli*. The urogenital swab cultured *Actinobacillus sp*.

- In March 2007, wild female 61 had a USG of 1.030, pH 8.5, that was positive for protein (3+), <1 WBC/hpf and <1 RBC/hpf with bacteria (1+) and struvite (1+) crystals seen on sediment exam and was negative for *Treponema*. *Brackiella oedipus*, *Corynebacterium pilosum* and non-haemolytic *E.coli* were cultured on urogenital swab. No swab of the rectal opening of the cloaca swab was obtained.

- In June 2005, wild female 66 had a USG of 1.015, pH 8.5, that was positive for protein (1+), 5 WBC/hpf with 1+ bacteria seen on sediment exam and was infected with *Treponema*. On urogenital swab *Klebsiella oxytoca* and *Enterobacter cloacae* were cultured. Swabs from the rectal opening of the cloaca revealed a heavy mixed aerobic growth, and moderate anaerobic growth.

- In November 2005, wild male 68 had a urine specific gravity (USG) of 1.033 which was turbid, pH 7.5, positive for protein(1+), blood(1+) >25 white blood cells(WBC)/hpf and >3 RBC/hpf, and bacteria (1+) were present on sediment exam. *Corynebacterium pilosum* was cultured from the urogenital and rectal opening of the cloaca in this individual. *Actinobacillus* sp. *Pasteurella*, *Clostridium glycolicum*, and *Prevotella* were also cultured from the urogenital sinus and this individual had a *Treponema* infection.

Statistical analysis using SPSS® (Chicago, Illinois) Version 15 for Windows was undertaken. However, due to the small sample size of 33 individuals only the minimum, maximum, mean and

standard deviation were recorded. Statistical analysis including comparison of cohorts via a one-way ANOVA was also performed using SPSS® (Chicago, Illinois). The most significant factor though to potentially alter urine values was the presence of *Treponema* infection. *Treponema* infection in male Gilbert's potoroos presents as a moderate-severe balanoposthitis with an associated green tenacious discharge. Although no serological response could be detected using the *Treponema pallidum* particle agglutination (TPPA), Enzyme immunoassay (EIA), and the non-specific Rapid plasmid reagin (RPR) test, given the severity of associated clinical signs it was decided to examine urine to check for differences between infected and non-infected individuals. Comparison of origin (captive compared to wild individuals) and sex (females compared to males) cohorts was undertaken. Age comparisons (sub-adults compared to adults) were not undertaken as there were only three sub-adults sampled.

Results

The results of urinalysis are found in Table 2. The volume of urine collected free-catch mid stream into a sterile urine container varied between 0.2mL and 7mL. Apart from Urine Specific Gravity (USG) and pH, analytes were qualitatively or quantitatively described. For example urine was described as clear in 86.2% of samples (25/29), cloudy in 6.5% of samples (2/29) and turbid in 3.4% of samples (1/29). All 29 samples were yellow in colouration (100%). The presence of bilirubin, ketones, SSA ppt, urothelial cells, and mucous threads was assigned to either be 0 (not present) or 1 (present). Urobilinogen was reported as 0 (negative) or 1 (normal). Therefore only minimum and maximum values were recorded for these analytes. Other analytes including glucose, blood, protein, sperm, bacteria, crystals and fungal elements were assigned to be 1 (present in mild amounts), 2 (present in moderate amounts) or 3 (present in heavy/high amounts). The remaining analytes including white blood cell count (WBC) and red blood cell count (RBC) were described according to the number of cells observed per high power field under light microscope.

Glucose was present in mild amounts (1+) in 6.9% of samples (2/29) (6.9%) and in moderate amounts (2+) in 3.4% of samples (1/29). No associated ketonuria was present in these samples, although ketones were present in 3.4% of samples (1/29). Bilirubin was present in 6.9% of samples (2/29). Blood was evident in a surprisingly high number of samples 34.5% of samples (10/29). Five samples had mild amounts (1+), two samples had moderate amounts (2+) and three samples had heavy amounts (3+). Protein was also found in 72.4% of samples (21/29) Fifteen samples had mild amounts (1+), four had moderate amounts (2+) and two had heavy amounts (3+).

Twelve samples 41.5% had normal values for urobilinogen. Five samples were positive for SSA ppt 17.2%. While only one sample contained urothelial cells and mucous threads 3.4%. Ten samples had sperm 34.5%, nine of which had a mild number (1+), with one having a moderate level (2+). Six samples had bacteria 20.7%, three samples had a mild number (1+), two had a moderate number (2+) and one had a high number (3+). One sample was found to have fat globules 3.4% and one sample 3.4% was found to have a high number of fungal elements.

Crystals found included calcium oxalate in 10.3% of samples (3/29) and struvite crystals in 34.5% of samples (10/29). Additionally 6.9% of samples (2/29) had concurrent calcium oxalate and struvite crystals found on microscopic examination of urine sediment.

Tests of normality conducted in SPSS® (Chicago, Illinois) included the Kolmogorov-Smirnov statistic, a histogram, detrended normal Q-Q plot and a boxplot. USG and pH values were found to be normally distributed. Other variables were descriptive as such statistics were not calculated.

Figure 2. Urinalysis results in Gilbert's potoroo

Analytes		Mean	Minimum	Maximum	Standard Deviation
Urine specific gravity	USG	1.02115	1.004	1.039	0.009306
pH	pH	6.981	6	8.5	0.9454
Glucose			0	3	0.801
Bilirubin			0	1	0.192
Ketone			0	1	0.192
Blood			0	3	1.111
Protein			0	2	0.669
Urobilinogen			0	1	0.492
Sulphosalicylic acid	SSA ppt		0	1	0.32
White blood cell count/hpf	WBC	1.22	0	7	1.188
Red blood cell count/hpf	RBC	4.37	0	70	2.589
Urothelial cells			0	1	0.192
Mucous threads			0	1	0.192
Sperm			0	3	0.747
Bacteria			0	3	0.792
Crystals			0	3	0.92
Fat globules			0	1	0.192
Fungal elements			0	3	0.577

Treponema infection in relation to the pH, protein, USG, presence of bacteria and crystals, number of WBC/hpf and the number of RBC/hpf were then examined using a one-way ANOVA in SPSS® (Chicago, Illinois). No significant differences were found in the above analytes in *Treponema* infected compared to non –*Treponema* infected individuals.

Sex effects on urinalysis values were also not significant when the pH, protein, USG, presence of bacteria and crystals, number of WBC/hpf and the number of RBC/hpf was examined using a one-way ANOVA in SPSS® (Chicago, Illinois).

Origin effects were evident in captive compared to wild individuals. Wild individuals had significantly greater values for pH (p=0.012), presence or absence of crystals (p=0.000), and presence or absence of bacteria (p=0.000). In fact, only the wild population had evidence of bacteria and crystals on microscopic examination of the urine sediment.

Discussion

Overall the urinalysis values obtained do not seem to substantially differ from those reported by Canfield *et al*. (1989) in the koala. However a few analytes such as the USG were considerably lower in the Gilbert's potoroo. Samples from healthy koalas had a mean specific gravity of 1.087 (range 1.062-1.135), while samples in healthy Gilbert's potoroo had a mean specific gravity of 1.021 (range 1.004-1.039) much closer to the typical values of companion animals. A healthy hydrated dog should have a USG between 1.015 and 1.045 (Kahn and Line 2005). The minimum value of 1.004 in Gilbert's potoroo was found in a captive sub-adult male. In companion animals this value would indicate excretion of urine more dilute than glomerular filtrate which requires

kidneys to perform metabolic work to produce hypotonic fluid in the distal tubules. This is not suggestive of renal failure but rather a primary polydipsia, central diabetes insipidus and conditions causing tubular insensitivity to vasopressin (ADH) (Kahn and Line 2005). A USG <1.008 can also occur in healthy animals that are excreting surplus fluid to maintain homeostasis, as in primary polydipsia (Thrall 2004). Repeat sampling of this individual revealed a USG of 1.021 however since the initial value did not seem to be of clinical significance it was included in the reference range, as no other indications of renal disease were apparent. The reason for the higher USG value in koalas as compared to the Gilbert's potoroo could be associated with the koala's adaptation to a diet of *Eucalyptus* leaves with variable but low water content. It is also interesting that urine remained concentrated in koalas that were administered fluid supplementation (Canfield *et al*. 1989). Suggesting adaptation of physiological mechanisms associated with urine excretion.

pH values in the koala varied depending on the method of diagnosis (Canfield *et al*. 1989), but were always more acidic than those of the Gilbert's potoroo. A pH of 5 was consistently returned with Labstix whereas Whatman's pH paper gave values ranging from 4.5-5.5. The Gilbert's potoroo samples were analysed with Multistix® Reagent strips (Bayer, Pymble, Australia), similar to Labstix, and a mean pH of 6.981 (range 6-8.5) was found. The variance between the two marsupial species is assumed to be dietary related, although the testing methodology could also account for some of the observed difference.

In the Gilbert's potoroo glucose was present in small amounts (1+) in 6.9% of samples (2/29) and in moderate amounts in 3.4% of samples (1/29). However, these values when analysed in conjunction with biochemistry could not be correlated to individuals with hyperglycaemia. However, an earlier case of hyperglycaemia, resulting in the appearance of glucose in the urine, which would have been stored in the bladder prior to specimen collection, could explain these results. This could certainly have occurred in wild individuals, which when trapped overnight could have developed an intermittent stress-related hyperglycaemia, which exceeded the renal threshold and led to glycosuria. Other possibilities include hereditary and acquired defects in proximal renal tubular glucose transport (Thrall 2004). No associated ketonuria was present in these samples, ketones were only present 3.4% of samples (1/29).

In studies of the koala undertaken by Canfield *et al*. (1989), urinalysis was negative for glucose, blood and bilirubin, and protein readings varied from 0.3 g/L to over 20 g/L. In the Gilbert's potoroo, blood was evident in a surprisingly high number 34.5% of samples (10/29) as was protein in 72.4% of samples (21/29). A positive occult blood test on a urinary reagent strip typically results from haemorrhage in the urinary tract, haemoglobin or myoglobinuria (Thrall 2004). Haemorrhage in the urinary tract was confirmed by the finding of red blood cells on sediment exam. This may have been related to the presence of *Treponema* infection in 62.1% of samples (18/29) as associated preputial ulceration was commonly reported. Proteinuria is also commonly seen in association with haemorrhage, inflammation or renal tubular degeneration. The sulpahsalicyclic acid (SSA ppt) qualitative test for protein was also conducted. This test was undertaken as false positives are common using reagent strips especially if the urine is concentrated. Haematuria will also falsely elevate the SSA ppt level. The SSA ppt test was positive in 17.2% of samples (5/29). This is considerably lower than the 72.4% of samples which were positive for protein using the reagent strip. Therefore the finding of proteinuria in those samples which had the presence of occult blood and those with evidence of *Treponema* infection is not surprising. However, fifteen samples were positive for protein on reagent strips yet negative for SSA ppt. Although the concurrent finding of blood may account for another five of these positive reagent test strip samples, the urine of the Gilbert's potoroo like the koala Canfield *et al*. (1989), may contain unidentified interfering substances. As such when interpreting protein values from a reagent strip the sulpahsalicyclic acid (SSA ppt) qualitative test for protein should also be undertaken.

Like koalas, the urine of Gilbert's potoroos contained amorphous crystals, bacteria, sperm, and lipid droplets, yet lacked casts. Bacteriology findings in koalas isolated *Klebsiella* sp.,

Streptococcus spp., *Staphylococcus* sp. and *Escherichia coli* but not in numbers considered to be significant. Gilbert's potoroos routinely returned urogenital cultures with *Actinobacillus* sp., *Corynebacterium pilosum*., *Pasteurella* sp., *Prevotella* sp., *Clostridium glycolicum*, and *Porphyromonas asaccharolytica* predominating. *Staphylococcus sp.*, *Klebsiella* sp., *Streptococcus* and non-haemolytic *E.Coli* were present in lower numbers (Chapter 5.3.2.1). The tendency for anaerobic growth in the Gilbert's potoroo was thought to favour the proliferation of *Treponema* and lead to subsequent pathology. Furthermore the presence of bacteria with urease producing properties (*Klebsiella, Proteus* spp. and *Staphylococcus* sp.) with a concurrent crystalluria is more likely to lead to pathologic consequences and the development of uroliths.

Comparison of cohorts

The lack of crystalluria and bacteriuria found in the captive colony is significant given the prior deaths from renal oxolosis which was presumed to be familial in the captive colony. No offspring of the originally affected individuals remain in the captive colony. However, the current prevalence of renal oxalosis in the wild population remains unknown. Urinary glycolate testing continues to be undertaken opportunistically, however blood is only routinely collected for haematology. Both of these samples are collected by DEC staff as part of their standard morphometric analysis and health profile reporting of the population. The only population blood biochemistry testing that has been undertaken was reported in Appendix 1. The results of this analysis found a statistically significant increase in urea, creatinine and phosphorus in the wild compared to the captive population. The presence of crystalluria and bacteriuria only in the wild population in conjunction with an elevation in renal biochemical parameters suggests renal function in the wild population is sub-optimal and warrants further investigation.

In this study no glycolate testing was undertaken. However opportunistic free catch collection of urine from 33 individuals over the course of the study revealed four individuals to have urine findings consistent with a urinary tract or preputial infection. Three of these four individuals 75% had *Treponema* infection, with spirochaetes being present on dark field microscopy and associated moderate to severe levels of urogenital discharge. Of the total population sampled for urine collection 63.6% of samples (21/33) had *Treponema* infection, however for the purpose of creating a reference interval of values in healthy individuals the above four potoroos were excluded and so the true prevalence in relation to the calculated reference intervals was 62.1% of samples (18/29).

Whether the excluded urinalysis results are reflective of urinary tract or preputial/urogenital infection is debatable. However given that three of the four individuals were *Treponema* infected there is a high likelihood of urogenital infection contaminating urine findings which were collected by the mid-stream, free catch method. Cystocentesis or catheterization would be recommended to eliminate bias in future studies. Although the lack of sepsis in the field will makes these methods logistically difficult. However trends can be still be noted in the above four cases, the pH was alkaline, proteinuria is present in moderate to high levels, WBC and RBC are consistently elevated above the mean and the presence of bacteria in the sediment was found in all four individuals. These findings are similar to Canfield *et al*. (1989) where 3 free-living koalas with cystitis showed increased leukocytes and/or erythrocytes and protein levels. The presence of opportunistic invaders in the Gilbert's potoroos such as *Actinobacillus* spp. *Corynebacterium pilosum*, *Enterobacter*, *E.Coli, Klebsiella and Prevotella* spp. on urogenital culture of these individuals is also likely to be clinically significant given bacteria were seen in the urine sediment and that comparative studies (Chapter 5.1) have revealed the potential pathogenicity of these species.

When individual urine analytes, known to be markers of inflammation were examined for the purposes of creating a reference interval, no statistical differences were evident between *Treponema*-infected compared to non-infected individuals. Therefore, we have evidence of urinary tract or preputial infection in three individuals with severe balanoposthitis and/or urogenital lesions associated with *Treponema* infection, one case of urinary tract infection from which *Treponema* was not isolated (GP F61) and no statistical significance in urinalysis values between otherwise healthy *Treponema* infected and non-infected individuals.

Urogenital inflammation can be caused by a number of mechanisms including, infection, obstruction, nephrotoxins, ischaemia, trauma and others (Davidson *et al.* 1988). The wild female GP F61 which was negative for *Treponema,* despite having evidence of urogenital inflammation on urinalysis had a concurrent bacteriuria and crystalluria. Culture results from the urogenital swab from this individual also revealed potentially pathogenic microbes which were likely to be the cause of the observed inflammation. The other three individuals with *Treponema* infection also had the presence of potentially pathogenic microbes on urogenital culture. As such, a mixed bacterial population, containing urease-producing bacteria could have led to the observed clinical signs and urinalysis findings. Certainly the results from such a small sample size should be interpreted with care, however the presence of abnormal urinalysis results indicative of urinary tract or preputial infection in three individuals infected with *Treponema*, all of which had evidence of severe balanoposthitis and/or urogenital lesions does seem clinically significant. When data is analysed from Chapter Five and Six, which discuss *Treponema* infection, in conjunction with histopathology findings there seems to be a strong causative relationship between *Treponema* and ulcerative hyperkeratosis of preputial skin. These lesions appear identical to those found in rabbits with venereal spirochetosis (Cunliffe-Beamer and Fox 1981). Therefore *Treponema* infection appears potentially pathogenic in causing urinary tract inflammation and infection.

Conclusion

Urinalysis values are described for Australia's most critically endangered mammal, the Gilbert's potoroo. Significant differences between origin (captive or wild individuals) were found, warranting further analysis of wild individuals' renal function. Sex, and presence or absence of *Treponema* infection did not significantly alter urine values. However, clinical cases of urogenital infection which resulted in abnormal urinalysis results demonstrated *Treponema* to be potentially pathogenic.

Therefore, in cases of presumed urogenital inflammation, *Treponema* screening should be undertaken via swabbing the urogenital area with; one wet swab for microbiological culture and dark field microscopy to detect spirochaetes and, another dry swab which should be submitted for *Treponema* PCR to the Department of Food and Agriculture in Kensington, Western Australia. Cystocentesis or catheterization is the preferred technique to obtain a sterile urine sample, but if not logistically possible a mid-stream free catch sample should be collected and interpreted cautiously. Urine sediment examination should be undertaken, followed by urinary glycolate testing following if calcium oxalate crystals are found during the sediment examination. Further diagnostics may include radiographic and ultrasound interpretation of the urogenital system for the presence of uroliths.

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APPENDIX 15: Cryptococcosis in Gilbert's (*Potorous gilbertii*) and long-nosed (*Potorous tridactylus*) potoroo.

Published in the Journal of Zoo and Wildlife Medicine December 2007.