Health and disease status in a threatened marsupial, the quokka (*Setonix brachyurus*)

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Declaration

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 educational institution.

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Abstract

Between 1901 and 1931, there were at least six anecdotal records of disease outbreaks in mainland quokkas (*Setonix brachyurus*) that were associated with mass. This time period pre-dates the arrival of the red fox (*Vulpes vulpes*). Despite these outbreaks, little or no research has been carried out to establish health and disease baseline data of the fragmented and scattered, extant populations. Epidemiological data was determined for a range of potential pathogens, and established physiological reference intervals of apparently healthy, wild quokkas on Rottnest Island and mainland locations. There were significant differences between Rottnest Island and mainland quokkas. Rottnest Island animals had haemograms with mark evidence of oxidative injury and bone marrow response consistent with a regenerative normocytic hypochromic anaemia. Except alkaline phosphatase (ALP), all blood chemistry analytes where higher in mainland animals, with particular emphasis on creatine kinase (CK), alanine amino transferase (ALT), aspartate amino transferase (AST) and vitamin E. Some other key findings include a widespread presence of a novel herpesvirus (MaHV-6), the recovery of *Cryptococcus neoformans* var. *grubii* from quokkas in highly altered ecosystems on Rottnest Island, and new *Salmonella* spp. serovars in Rottnest Island quokkas. Atypical lymphocytes resembling those in proliferative disorders of the lymphoid and haematopoietic tissues in other species were observed in blood smears of animals on Rottnest Island but not on the mainland. The presence of potentially-pathogenic organisms is likely to increase synergistic effects of ongoing and future threats (e.g. habitat clearing, climate change), and could increase quokka extinction risk. Disease surveillance would make a valuable contribution to Recovery Plans for the quokka, enabling preparedness for a rapid response if clinical disease is to happen, and to manage populations in a more integrated way.

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Disclaimer

This PhD thesis consists of chapters that have been prepared as stand-alone manuscripts. These manuscripts (Chapters 2, 4, 5, 6, and 7) are being prepared for future submission. As a consequence, there may be some repetition between chapters. References are presented together at the end of the thesis.

Conference proceedings and publications arising from this work

Conference proceedings and other works

"Disease surveillance in quokkas as a conservation tool". Australasian Wildlife Management Society: 28th Annual Conference, Wildlife Management in a Changing Environment, 23-26 November 2015. Perth, Western Australia.

"Infectious Agents in Quokkas". Infectious Diseases Seminar 2013. Telethon Institute for Child Health Research. 6th February 2013. Perth, Western Australia.

"Preliminary studies on Cryptococcus species in free-ranging quokka (*Setonix brachyurus*) a threatened marsupial in Western Australia". Wildlife Disease Association-Australasia: Annual Conference Proceedings. 23rd-28th September 2012. North Stradbroke Island, New South Wales.

Papers for publication

Martinez-Perez, P.A., Hyndman, T.H., Fleming, P.A., Vaz, P.K., Ficorilli, N.P., Wilks, C. *A widespread novel gammaherpesvirus in apparently healthy free-ranging quokkas (Setonix brachyurus): a threatened and endemic wallaby of Western Australia*. ***In preparation***

In this paper, the molecular detection and phylogenetic placement of the sixth herpesvirus in macropods are presented. Important epidemiological data such as prevalence and geographical distribution are reported. A thorough statistical analysis to determine possible associations between the presence of this new gammaherpesvirus and changes in the haematology and blood chemistry profiles of the infected animals was carried out. Management and conservation implications of the presence of this virus are discussed.

Martinez-Perez, P.A., Hyndman, T.H., Fleming, P.A. *An evaluation of the association between the presence of Salmonella and the health of S. brachyurus from Rottnest Island and the mainland of the South-West of Western Australia*. ***In preparation***

In this paper, new and updated epidemiological data (e.g. prevalence, serovar abundance and richness, distribution) for *Salmonella* in *S. brachyurus* on Rottnest Island and mainland Western Australia are presented. A new risk factor (microfilariae) for *Salmonella* infections in the Rottnest Island quokka is presented and analysed. Most importantly, evidence of an effect of *Salmonella* on the haematology and blood chemistry profiles is presented, suggesting that *Salmonella* infections in the quokka are diseased entities. Management and conservation implications of *Salmonella* in the Rottnest Island quokka are discussed.

Martinez-Perez, P.A., Hyndman, T.H., Fleming, P.A. *Isolation of Cryptococcus neoformans* var. *grubii (serotype A) and Cryptococcus magnus from the nasal lining of free-ranging quokkas on Rottnest Island*. ***In preparation***

In this paper, a comparative study between Rottnest Island and mainland subpopulations, on the recovery, isolation and molecular detection and identification of yeast is presented. Specific emphasis is given to the isolation and characterisation of *C. neoformans* var. *grubii* and *C. magnus*. Valuable epidemiological data such as prevalence, and distribution are provided, and a thorough statistical analysis aimed to detect possible associations between the presence of *C. neoformans* var. *grubii* and *C. magnus* and changes in the haematology and blood chemistry of infected animals is presented. The possible public health implications of the presence of *C. neoformans* var. *grubii* on Rottnest Island are discussed. Management and conservation implications of the presence of these yeast are considered.

Martinez-Perez, P.A., Fleming, P.A., Hyndman, T.H. *Haematology and blood chemistry of free-ranging quokkas (Setonix brachyurus) from Rottnest Island and selected locations on the mainland of Western Australia*. ***In preparation***

In this paper, the first blood chemistry reference intervals, and the most comprehensive haematology study and reference intervals of free-ranging *S. brachyurus* on Rottnest Island and mainland Western Australia are presented. The detection of atypical lymphocytes in Rottnest Island animals, resembling those in proliferative disorders of the lymphoid and haematopoietic tissues is discussed. Similarly, other factors possible involved in the seasonal anaemia experienced by animals on Rottnest Island are explored. A comparative study of vitamin E in plasma

between animals in captive conditions and their wild conspecifics is given. The molecular detection of *Babesia* sp. and *Theileria* sp. are discussed.

Martinez-Perez, P.A., Fleming, P.A., Lee, J.G., Wilks, C.R., Woods, R., Hyndman, T.H. *Diseases and pathogens reported in the quokka, Setonix brachyurus (Quoy & Gaimard 1830) (Marsupialia:Macropodidae) with notes on its ecology, status and threats: a review*. ***In preparation***

A review paper of the disease entities and pathogens reported in quokkas, combined with material on the species ecology, conservation status and threats.

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List of Abbreviations

AHL: Animal Health Laboratories

ALB: Albumin

ALP: Alkaline Phosphatase

ALT: Alanine Aminotransferase

ARWH: Australian Registry of Wildlife Health

AST: Aspartate Aminotransferase

ATL: Adult T-cell leukaemia

AVs: Arboviruses

BASO: Basophils

BCE: Brown Colony Effect

BCI: Body Condition Index

BFV: Barmah Forest virus

BILT: Total Bilirubin

bp: base pairs

BSA: Bird Seed Agar

C: Citrate

CALC: Calcium

CALP: Corticosteroid alkaline phosphatase

CFU: Colony Forming Unit

CHCM: Corpuscular Haemoglobin Concentration Mean

CHOL: Cholesterol

CI: Confidence Interval

CK: Creatine Kinase

CLSI: Clinical and Laboratory Standards Institute

CNS: Central nervous system

CREAT: Creatinine

CRT: capillary refilled time

CUAT: Christensen's Urea Agar Test

CWR: Critical Weight Range

DAFWA: Department of Agriculture and Food of Western Australia

DFTD: Devil Facial Tumour Disease

DNA: Deoxyribonucleic Acid

DPOL: DNA-dependant polymerase

dNTPs: Deoxynucleotide triphosphates

DPaW: Department of Parks and Wildlife

DSE: Delayed Secondary Enrichment

DW: Distilled Water

EDTA: Ethylene Diamine Tetraacetic Acid

EHV-1: Equine herpesvirus 1

EMCV: Encephalomyocarditis virus

EOS: Eosinophils

ES: Effect Size

FWPV: Fowlpox virus

GGT: Gamma-glutamyl Transferase

GLOB: Globulin

GLUC: Glucose

h: Hour

HGB: Haemoglobin

HI: Haemagglutination Inhibition

HTLV-1: Human T-Cell Leukaemia Virus 1

I: Indole

IFCC: International Federation of Clinical Chemistry

IFN-γ: Interferon gamma

IL: Interleukin

ISIS: Species Information System

ITS: Internal Transcribed Spacer Region

IUCN: International Union for Conservation of Nature

JF1: Northern Jarrah Forest Biogeographical Region

JF2: Southern Jarrah Forest Biogeographical Region

KoRV: Koala retrovirus

L: Lactose

LCAT: Latex cryptococcal agglutination test

LD: Lysine Decarboxylase

LYMPH: Lymphocytes

MaHV: Macropodid herpesvirus

MCHC: Mean Corpuscular Haemoglobin Concentration

MCV: Mean Corpuscular Volume

MHA: Mueller-Hinton Agar

min: minute/s

MONO: Monocytes

MORV: Mammalian orthoreovirus

MR: Methyl Red

MWU: Mann-Whitney U test

NaCl: Sodium Chloride

NA: Nutrient Agar

NB: Nutrient Broth

N:C: nuclear:cytoplasm ratio

NEUT: Neutrophils

nMDS: non-metric Multidimensional Scaling

nPCR: nested Polymerase Chain Reaction

nRBCs: nucleated Red Blood Cells

NTM: Non-tuberculous mycobacteria

NSW: New South Wales

OR: Odds Ratio

ORBV: Orbivirus

ORBVs: Orbiviruses

ORV: Orthoreovirus

ORVs: Orthoreoviruses

PBCM: Peripheral Blood Cell Morphologies

PBS: Phosphate buffered saline

PCR: Polymerase Chain Reaction

PCV: Packed Cell Volume

PhaHV: phascolarctid herpesvirus

PHOSP: Phosphorus

PLT: Platelet Concentration

PV: Poxvirus

PVs: Poxviruses

PW: Peptone Water

QLD: Queensland

RBC: Red Blood Cell Count

rDNA: ribosomal Deoxyribonucleic Acid

RNA: Ribonucleic Acid

rRNA: ribosomal Ribonucleic Acid

RP: Recovery Plan

RPs: Recovery Plans

RRV: Ross River virus

RT-PCR: Real Time Polymerase Chain Reaction

RV: Rappaport Vassiliadis

RVs: Reoviruses

SABC: State Agricultural and Biotechnology Centre

SD: Standard Deviation

SDA: Sabouraud Dextrose Agar

sec: seconds

SOP: Standard Operation Procedure

TP: Total Protein

TRUV: Trubanaman virus

U: Urea Hydrolysis

UREA: Urea

vit.: Vitamin

VP: Voges-Proskauer Test

WA: Western Australia

WBC: White Blood Cell Count

WHO: World Health Organization

XLD: Xylose Lysine Deoxycholate

1. General Introduction

According to the 2012 International Union for Conservation of Nature (IUCN) Annual Report, of the 63,837 species of vertebrates and plants that have been assessed, 19,817 are threatened with extinction, of which 25% are mammals (IUCN 2015). This report also indicates that over 500 species are considered possibly extinct or extinct in the wild and this number is likely an underestimate. It has been estimated that between 1970 and 2005, an overall decline of 30% in 1,698 vertebrate species occurred worldwide (Loh *et al.* 2008). In Australia, 29 species of mammals are now extinct. This is more than any other continent (Ceballos & Ehrlich 2002 ; Johnson 2006 ; McKenzie *et al.* 2007 ; Woinarski, Burbidge, & Harrison 2014). A further 57 species, which represents 18.3% of the total number of species in Australia are considered threatened (Woinarski, Burbidge, & Harrison 2014). Of these, those with body mass ranging from 35 to 5,500 g (i.e. critical weight range, CWR), generally herbivorous and living in low rainfall regions, have been most vulnerable (Burbidge & McKenzie 1989 ; Johnson & Isaac 2009 ; Johnson, Burbidge, & McKenzie 1989 ; Short & Smith 1994). The most common factors that are considered to contribute, either directly or indirectly, to loss of Australia mammal species include habitat modification, introduced species (competitors and predators), modification of fire regimes, climate change, over exploitation, inbreeding depression. Infectious diseases have also played a role in wildlife population reductions and extinctions; however, the role of diseases has generally been overlooked.

As early as the 1930's, Aldo Leopold, a prominent figure in wildlife management in North America stated that "the role of disease in wildlife conservation has probably been radically underestimated" (cited by Spalding & Forrester 1993). This view has persisted through the years. For instance, Daszak et al. (2000) in their retrospective study of vertebrate translocations, as part of wildlife management projects, determined that of more than 2,000 translocations carried out between 1973 and 1986 in Australia, Canada, New Zealand and the United States combined, more than 70% of those projects did not investigate the causes of mortality after translocation, including disease-related causes of mortality. More recently, Pedersen et al. (2007) used data from the 2006 IUCN Red List to determine that published studies on infectious agents in populations of wild mammals were available for only <40% of the world's most threatened artiodactyls, primates and carnivores, while 25% of species that were threatened by infectious agents had little or no

published information regarding the presence of such infectious agents at a population level.

Regrettably, there are a number of factors that have trivialised the importance of diseases in wildlife. First, some of the available data implicating infectious diseases in population declines or extinctions are largely anecdotal. Second, baseline data concerning infectious diseases in wildlife is scant. Third, most of the non-anecdotal data correspond to studies that were carried out in response to a mortality event, in which case research is for the most part focused on identifying the organism and elucidating its mechanisms of disease, and perhaps its environmental associations, but too frequently there is no interdisciplinary work with the fields of science that look after wildlife population demographics (e.g. ecology). Consequently, only rarely has the impact of mortality events on population status been explored. Fourth, that a great deal of non-anecdotal data of infectious agents/diseases in wildlife comes from investigating diseases important to human health and domestic animals, thus leaving out their relevance and impact on the wildlife species that were studied in the first place. Fifth, there is still research undertaken in wildlife, that although focused on infectious organisms, limited their results to the classification of the organism, with little or no consideration for the immediate or future impact on the health of the species in question (Austen *et al.* 2009 ; Bennett & Hobbs 2011 ; Clark & Spencer 2007 ; Hart, Bradshaw, & Iveson 1985, 1986 ; Lozano *et al.* 2015 ; Mora *et al.* 2015 ; Paparini *et al.* 2011 ; Smith, Clark*, et al.* 2008). And sixth, is the persistent view of many ecologists and wildlife biologists that do not recognise disease as a significant factor in wildlife management. This posture has usually been defended by reasons such as the high cost of health studies, the inconvenience of the logistics needed to assess the health of the animals, a decrease in recapture rates due to the "invasive" procedures commonly carried out (e.g. venipuncture); and the arguments that animals with compromised fitness due to disease would eventually be removed by predators, and the absence of overt signs of disease is sufficient evidence supporting the absence of disease.

Despite these hurdles, evidence suggests that infectious diseases do indeed play a significant role in the persistence of wildlife populations and can drive species to extinction (Aguirre & Tabor 2008 ; Daszak & Cunningham 1999 ; Hartigan *et al.* 2011 ; Leendertz *et al.* 2006 ; Pedersen *et al.* 2007 ; Schloegel *et al.* 2006). Using data from the 2006 IUCN Red List, Smith et al. (2006) determined that infectious diseases were within the top five processes contributing, or that have contributed, to global species extinction. Although these results indicated that the degree of contribution (direct or indirect) was

less than 4% of known species extinctions since 1500, and less than 8% of 2,852 critically endangered plants and animals, this work did show the first numerical evidence of the impact of disease at a global scale. In support of the findings of Smith (2006), the work on mammals by Pedersen et al. (2007) that also used data from the 2006 IUCN Red List, determined that of the reported infectious agents threatening wild mammals, viruses and bacteria were the most common, although other groups were also mentioned (e.g. protozoans and fungi), and that organisms transmitted by direct contact were more likely to be involved in extinction risk than those with other forms of transmission. There are a number of cases in which infectious diseases have been implicated in population declines of mammals, birds and amphibians (Table 1-1) For example, white-nose syndrome caused by *Geomyces destructans*, has been responsible for drastic declines in insectivorous bats in North America with an estimated 5.5 million deaths since 2007 (Lorch *et al.* 2013 ; Thogmartin *et al.* 2012), while chytridiomycosis caused by *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*, has been responsible for mass declines in frogs and salamanders (Martel *et al.* 2013 ; Phillott *et al.* 2013) with over 200 species apparently being threatened worldwide (Skerratt *et al.* 2007).

Table 1-1 Cases in which infectious diseases have been implicated in population declines and species extinction.

Animal species	Location	Disease identified	Impact	Reference
European wild rabbit (*Oryctolagus cuniculus*)	Spain	*Rabbit haemorrhagic disease virus*	~60% pop. decline	(Moreno *et al.* 2007)
Gorilla (*Gorilla gorilla*) and Chimpanzee (*Pan troglodytes*)	Gabon/Republic of Congo border	*Ebolavirus* sp.	85% pop. decline	(Leendertz *et al.* 2006)
Various avian species	North America	*West Nile virus*	50-100% pop. mortality	(LaDeau, Kilpatrick, & Marra 2007)
Wild common carp (*Cyprinus* sp.)	Canada	Koi herpesvirus	> 25,000 deaths	(Garver *et al.* 2010)
Santa Catalina Island fox (*Urocyon littoralis catalinae*)	North America	*Canine distemper virus*	~85% pop. reduction	(King, Duncan, & Garcelon 2014 ; Timm *et al.* 2009)
Insectivorous bats	North America	white-nose syndrome *Geomyces destructans*	~5.5 million deaths	(Lorch *et al.* 2013 ; Thogmartin *et al.* 2012)
frogs and salamanders	Worldwide	chytridiomycosis caused by *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans*	over 200 species threatened	(Skerratt *et al.* 2007)
Tasmanian devil (*Sarcophilus harrisii*)	Australia	Tasmanian devil facial-tumour disease	>90% pop. decline	(McCallum 2008 ; McCallum *et al.* 2007)
Christmas Island pipistrelle (*Pipistrellus murrayi*)	Australia	*Trypanosoma lewisi*	Extinction	(Woinarski, Burbidge, & Harrison 2014)
Endemic Hawaiian honeycreepers	Hawaii	*Avipox* and *Plasmodium* spp.	Extinction	(Atkinson & LaPointe 2009)
South Island saddlebacks (*Philesturnus carunculatus carunculatus*)	New Zealand	*Avipox* and *Plasmodium* spp.	%60 decline	(Alley *et al.* 2010)

Extinction has also been attributed to infectious diseases. Some of the more significant cases include, the annihilation of the last free-ranging colony of the black-footed ferret (*Mustela nigripes*) in North America, due to *Canine distemper virus* infection (May 1986 ; Thorne & Williams 1988), and the disappearance of *Partula turgida*, a species of snail, as a result of a microsporidian parasite infection (Cunningham & Daszak 1998 ; Schloegel *et al.* 2006). Similarly, *B. dendrobatidis* has been suggested by different authors as the driving force of the greatest extinction wave in modern history due to an infectious disease, with a conservative number of over 100 different species of frogs possibly extinct worldwide (Kriger & Hero 2009 ; Skerratt *et al.* 2007 ; Vredenburg *et al.* 2010).

Locally, Australian native fauna have also experienced the impact of infectious diseases. Perhaps the two most important cases are the confirmed disappearance of the Australian sharp-snouted day frog (*Taudactylus acutirostris*) due to infection with *B. dendrobatidis* (Schloegel *et al.* 2006), and the drastic population crash (>90%) of the Tasmanian devil

(*Sarcophilus harrisii*) due to Tasmanian devil facial-tumour disease (McCallum 2008 ; McCallum *et al.* 2007). Some other Australian species extinctions and declines that were partly attributed to infectious diseases include the Tasmanian thylacine (*Thylacinus cynocephalus*) (possibly distemper and mange Guiler 1961 ; Paddle 2012), the Christmas Island rat (*Rattus macleari*) (Trypanosoma levisi infection Pickering & Norris 1996 ; Wyatt *et al.* 2008), and more recently, the brush tailed bettong (*Bettongia penicillata*) (Trypanosoma sp. Botero *et al.* 2013 ; Smith, Clark, *et al.* 2008). In addition to these examples, there seems to be strong anecdotal evidence that disease has been involved in mammal declines and extinctions in Western Australia (WA) since as early as 1875 (Abbott 2006).

Although the impact of disease on an individual or on a population is easy to recognise when there is obvious disability (e.g. bleeding, fractures, tumours, circling), or when mass mortalities occurred, disease can also be present in the absence of clinical signs. Subclinical disease can reduce overall fitness and can cause injury to specific organ systems. For instance, *Cowpox virus* infection in wood mice (*Apodemus sylvaticus*) and bank voles (*Muodes glareolus*), although asymptomatic, was responsible for reducing their reproductive output by at least 25% (Feore *et al.* 1997); while sage grouse (*Centrocercus urophasianus*) males infected with avian malaria (*Plasmodium pediocetti*) bred later in the season and copulated less frequently than uninfected males (Johnson & Boyce 1991). Similarly, studies have shown that the simple event of mounting an immune response to an antigen can be deleterious to the overall fitness of the animal or population. For instance, Ilmonen et al. (2000) observed that female pied flycatchers (*Ficedula hypoleuca*) with an activated immune response after immunisation with non-pathogenic antigens, had lower reproductive output than females that were not immunised. Supporting this, Hanssen et al. (2004) correlated a humoral immune response with compromised long-term survival in common eiders (*Somateria mollissima*).

There is evidence from indicating that stress plays a role in disease. Stress is strongly linked to disease, and has been suggested as a factor that exerts great influence on various diseases in wildlife, including toxoplasmosis in marsupials (Thompson, Lymbery, & Smith 2010), white nose syndrome in bats (Cryan *et al.* 2010), *Chlamydia* infection in koalas (Brearley *et al.* 2013), and chytridiomycosis in amphibians (Gabor, Fisher, & Bosch 2013). There is no universal definition of stress but in the context of animal health, it can be defined as the alteration of physiological homeostasis driven by a physiological, environmental or psychological stressor, or a combination of these (sensu lato Black 1994), and can cause significant downregulation of the immune system (i.e.

immunosuppression) among other effects. Immunosuppression might occur following long term exposure to endogenous glucocorticoids, which are released during periods of stress (Dhabhar & McEwen 1997 ; Padgett & Glaser 2003). Levels of these glucocorticoids can increase significantly due to persistent and increased predator presence (Polednik *et al.* 2008), climate fluctuations (Shultz & Kitaysky 2008) and human disturbance (Schmidt *et al.* 2009). Immunosuppression can be expressed as decreased leukocyte movement, decreased effector cell function, decreased cell-mediated immunity, and decreased humoral immunity. This increases susceptibility to infectious diseases and proliferative disorders, exacerbates the effects of an infection, increases or activates shedding of pathogens, and can reactivate latent pathogens (Friedman & Lawrence 2002 ; Hofer & East 2012). It is possible that as threats increase in magnitude and occur more rapidly, chronic stress may develop in wild populations, and with it greater exposure to endogenous glucocorticoids, which may lead to greater risk of immunosuppressive states and disease.

The possible outcomes or impacts in a mixed infection scenario are quite numerous and may include genetic, metabolic, behavioural and immunological impacts. However, studies on concomitant infection in humans and mice, which for the most part have focused on the interactions of protozoans and helminths with other groups of organisms (e.g. virus and bacteria), indicate that the most common outcome in hosts with mixed infections, is that in which one agent causes downregulation of the immune system, while the other agent or agents take advantage of this resulting immunosuppression, proliferate and eventually cause disease (Cox 2001). Some of the studied cases of this parasitic relation include concurrent infections with Epstein-Barr virus (*Gammaherpesvirinae*) and *Plasmodium falciparum* in humans. In this situation, the T-cell response of the malaria-infected host appears to lose control of the viral infection (Wedderburn *et al.* 1984). A second example involves the lethal effect of normally non-lethal strains of *P. chabaudi* and *P. yoelii* in mice infected with *Rotavirus C* (colloquially known as Rowson-Parr virus) (Cox, Wedderburn, & Salaman 1974) but not in non-infected *Rotavirus C* mice. In mice, suppression of Type 1 T helper cells (Th_1) following infection with *Fasciola hepatica* reduced the protective response against *Bordetella pertussis* (Brady *et al.* 1999). Similar outcomes where one infectious agent caused immunosuppression and in doing so favoured the proliferation of other agents, have been observed in wildlife. For instance, the dramatic mortality of Serengeti lions (*Panthera leo*) between 1994 and 2001 in which *Babesia* sp. infections were believed to have been intensified by the immunosuppressive effects of *Canine distemper virus* (Munson *et al.* 2008). A similar case is that of the South Island saddlebacks (*Philesturnus carunculatus carunculatus*) population in New Zealand, in which infection with *Avipoxvirus* is thought to have caused the right conditions

(immunosuppression) for *Plasmodium elongatum* (thought to be endemic in this species) to overrun the hosts immune response (Alley *et al.* 2010). Together with other factors (e.g. reduce heterozygosity), this coinfection is believed to have contributed to a 60% decline in the saddlebacks populations. According to Telfer et al. (2010), mixed infections appear to carry a more significant impact on infection risk in wildlife, than factors such as season and host age. Despite the limited studies in wildlife, there appears to be strong evidence from other mammalian hosts that call for a more systematic approach to the study of the effect of multiple infections on the health of native wildlife.

Disease has the potential to cause significant morbidity and mortality in wildlife, directly and/or indirectly, and could push species to significant population declines and even extinction. Because of the risk of potentially catastrophic effects of disease on the conservation status of Australian native wildlife, greater attention to disease surveillance and health monitoring as well as increased knowledge on the microbiome of these species is required.

In Australia, endangered species are commonly managed through execution of a Recovery Plan (RP), which represent a comprehensive review of the current conservation status and threats for a given species, highlighting gaps in knowledge that are relevant to the conservation of the species, and prioritising management options required to support the persistence of that given species. In WA, these conservation initiatives fall under the jurisdiction of the Department of Parks and Wildlife (DPaW). Twelve RPs for threatened native wildlife in WA (http://www.dpaw.wa.gov.au/platns-and-animals/threatened-species-and-communities/197-approved-recovery-plans), that were approved between 2003 and 2015, were reviewed (Table 1-2).

Table 1-2 Twelve approved Recovery Plans for 21 mammal species native to Western Australia.

Species	Binomial name	Year Approved	Disease a threat (priority)	Surveillance/health monitoring as a conservation action	Reference
Golden bandicoot	*Isoodon auratus*	2003	No	No	(Palmer, Taylor, & Burbidge 2003)
Golden-backed tree-rat	*Mesembriomys macrurus*	2003	No	No	(Palmer, Taylor, & Burbidge 2003)
Greater bilby	*Macrotis lagotis*	2006	No	No	(Pavey 2006)
Northern quoll	*Dasyurus hallucatus*	2010	Yes	Yes	(Hill & Ward 2010)
Rufous hare-wallaby (central mainland species)	*Lagorchestes hirsutus* spp.	2012	Yes	No	(Jacqueline 2012)
Rufous hare-wallaby (Bernier Island)	*Lagorchestes hirsutus bernier*	2012	Yes	No	
Rufous hare-wallaby (Dorre Island)	*Lagorchestes hirsutus dorreae*	2012	Yes	No	
Chuditch	*Dasyurus geoffroii*	2012	No	No	(Department of Environment and Conservation 2012a)
Black-footed rock wallaby	*Petrogale lateralis*	2012	Yes	Yes	(Pearson 2013)
Short-eared rock wallaby	*Petrogale brachyotis*	2012	Yes	Yes	
Monjon	*Petrogale burbidgei*	2012	Yes	Yes	
Nabarlek	*Petrogale corcinna*	2012	Yes	Yes	
Rothschild rock wallaby	*Petrogale rothschild*	2012	Yes	Yes	
Woylie	*Bettongia penicillata ogilbyi*	2012	Yes	Yes	(Yeatman & Groom 2012)
Plains mouse	*Pseudomys australis*	2012	No	Yes	(Moseby 2012)
Western barred bandicoot	*Perameles bougainville*	2012	Yes (High)	Yes	(Department of Environment and Conservation 2012b)
Burrowing bettong	*Bettongia lesueur*	2012	Yes (High)	No	
Banded hare-wallaby	*Lagostrophus fasciatus*	2012	Yes (High)	No	
Quokka	*Setonix brachyurus*	2013	Yes (Low)	Yes	(Department of Environment and Conservation 2013)
Western ringtail possum	*Pseudocheirus occidentalis*	2014	Yes	No	(Department of Parks and Wildlife 2014)
Numbat	*Myrmecobius fasciatus*	2015	Yes	Yes	(Department of Parks and Wildlife 2015)

In four of the 12 RPs examined (golden bandicoot/golden-backed tree-rat, greater bilby, chuditch and plains mouse), disease was not mentioned as a possible threat. Consequently, neither disease surveillance nor health monitoring were considered as part of the actions necessary to support the persistence of the species (except for the plains mouse RP). Of the remaining RPs that listed disease as a possible threat to the species, disease surveillance and/or health monitoring is recognised as important for six RPs involving 12 species (i.e. northern quoll, western barred bandicoot/burrowing bettong/banded hare-wallaby, numbat, rock wallabies, quokka, and woylie RPs), with three RPs giving disease surveillance and/or health monitoring a high priority, two considered these actions of medium priority, and one gave them a low priority.

Disease surveillance and/or health monitoring actions have been considered more frequently in recent RPs (2010 onwards), however, specific recovery actions were not always listed to address this (e.g. rufous hare-wallabies, western ringtail possums, and banded hare-wallabies, Table 1-2). Similarly, some RPs that listed introduction of disease into a population as an undesirable event, against which an emergency response should be planned, did not include disease surveillance and health monitoring (e.g. post-mortem examination) in the actions needed to design such a task. In some other cases, it was believed that disease posed a threat only to island populations but not to mainland populations. In addition, in all 12 RPs, whether disease is mentioned or not as an active or possible threat, or having played a direct or indirect role in the decline of the species, it appears that easy-to-observe events such as mortality and overt signs of disease (e.g. skin lesions, fractures, bleeding), are the defining factors by which disease is considered significant or not. This approach to disease ignores subclinical disease, which generally presents in the absence of obvious signs of disease, and can put individuals and populations at a selective disadvantage by synergistically increasing vulnerability to other threatening factors. Lastly, according to the RPs examined which covered 21 species, some disease and health baseline data appear to have been obtained for nine (43%) of these, either as part of the RPs actions or from other sources. Of these nine, only three (14%) have comprehensive disease and health baseline data. This means that 86% of species covered by these 12 RPs, do not have comprehensive health or disease baseline data available.

Recognising the limitations and impacts associated with the lack or absence of disease and health baseline data in native Australian wildlife is critical. Without doing this, the applicability of tools such as disease risk analyses would be compromised due to the absence of necessary information such as prevalence, incidence, and distribution of an

infectious agent. This can only be obtained through disease surveillance. Preparedness is also severely compromised and conservation actions would become reactionary in the event of an infectious disease outbreak. This would then lead to a less efficient and effective response when such response is feasible, and would increase the risk of biodiversity loss, particularly if an outbreak scenario involves a highly pathogenic and infectious agent. Uninterrupted disease surveillance and health monitoring are the only tools by which changes in prevalence, incidence, seasonality, geographical distribution and population distribution of a given infectious agent can be determined.

Unfortunately, disease surveillance in wildlife is characterised by a series of limitations. For instance, monitoring the health and disease status of wild populations is intrinsically more difficult than in other groups (e.g. domestic and captive animals) because wild animals can move long distances (e.g. migratory birds), populations can extend over large areas that not too unfrequently, have difficult access or are inaccessible all together, and certain procedures and measurements may need the use of sophisticated equipment and protocols (i.e. general anaesthesia). Furthermore, the availability of carcasses for post mortem examination is often hindered by factors such as, advance states of decomposition, scavenging, or simply not found. Consequently, disease surveillance and health monitoring could present as very costly initiatives due to logistic requirements. But, in addition to this, funding opportunities tend to prioritise research in other areas such as human health, climate change or livestock disease, over wildlife research (Vrbova *et al.* 2010).

Despite these inherent difficulties, it is now widely acknowledged that nations that carry out disease surveillance of their wildlife populations can expect to understand disease patterns, and therefore better protect not just their wildlife populations but human populations too. In the absence of disease and health baseline data in free-ranging populations of native Australian fauna, conservation actions would have to rely on theoretical models, historical data and isolated studies on the species of interest. This approach has severe limitations. This is because historical data on the impact of infectious disease on wild populations is anecdotal for the most part and isolated studies generally operate around the classification of their findings, neglect sampling multiple populations, and ignore the potential effects of such findings on the health of the animals. Similarly, theoretical models often come from species that are too taxonomically distant. To predict and respond in a proactive manner to the effects of disease on the health of Australian native fauna, it is necessary to understand that there is a continuous feedback between disease and other factors (e.g. land clearing, urban development, climate fluctuations,

predators, mix infection), where one predisposes to the other. Similarly, it is necessary to understand that the early identification of infectious agents facilitates appropriate conservation action, but it is only through ongoing disease surveillance and health monitoring that this can be achieved.

The following project was carried out in response to the absence of disease and health baseline data in free-ranging quokka populations, and to proactively push for a preventative management approach to disease in this vulnerable species.

1.1 The quokka (*Setonix brachyurus*) disease and health assessment project

The research project that is the subject of this thesis was undertaken between 2009 and 2013 and encompassed health and disease monitoring of free-ranging individuals from Rottnest Island, and from groups in three subpopulations on the southwest of mainland Western Australia: Northern Jarrah, Central Jarrah and Southern Forest. The main goal was to determine the current relative health (define as the absence of obvious anatomical and organic dysfunctions) and disease status of the animals at the time of sampling, and retrospectively explore the possible associations/correlations through statistical analyses, between the presence of some selected infectious agents and physical examination observations, as well as haematology and blood chemistry changes, if present. Due to the multifactorial nature of health and disease, this study included independent variables such as season and geographical location in the analyses. Additionally, given the pathogenesis of the infectious organisms studied (*Salmonella* spp. *Cryptococcus* spp., and herpesvirus) in which immunosuppression of the host plays a significant role in the establishment of clinical disease, underlying immunosuppression was always considered by examining the potential relationships with prevalence of these organisms.

Lastly, as part of the health assessment component, reference intervals for physiological parameters (haematology and blood chemistry) were constructed. This then allows a more objective tool to assess the health of free-ranging quokkas across a range of environmental conditions. Key objectives of this component included, to increase the number of potential covariates recorded, to increase statistical power for analyses by increased sample size, and to implement new and accepted statistical approaches, as determined by the International Federation of Clinical Chemistry and the Clinical and Laboratory Standards Institute (CLSI).

The findings of this research project aim at contributing to the knowledge of the microbiome of the quokka that could facilitate a quicker response in the event of an outbreak, and serve as baseline information to exercise defensive management against infectious agents and their potential impact on this threatened species, if clinical disease was to happen. Similarly, this study intends to contribute to the veterinary management of the quokka, in both wild and captive conditions, and be instrumental in further research on the species.

1.2 Aims of this research

To study and determine the general health of quokkas on Rottnest Island and individuals from three subpopulations on the mainland using a variety of diagnostic methods:

- General physical examination;
- Haematology and blood chemistry through automated analysers, with further manual differential of white blood cells and assessment of erythrocyte morphologies;
- Testing for *Salmonella* spp. through cultural methods on faecal samples and identification of isolates through serotyping using the White-Kaufmann-Le Minor scheme;
- Testing for *Cryptococcus* spp. through cultural methods on nasal swabs, and identification of *Cryptococcus* spp. isolates through Polymerase Chain Reaction (PCR) and sequencing;
- Testing for exposure to *Macropodid herpesvirus 1* (MaHV-1) and *Macropodid herpesvirus 2* (MaHV-2) by virus neutralisation and testing for presence of herpesviruses through Polymerase Chain Reaction (PCR) on blood samples and identifying herpesviruses by sequencing and phylogenetic analysis;
- Testing for intraerythrocytic parasites and trypanosomes by blood smear examination and PCR (followed by sequencing for identification);
- Screening faecal samples for nematode eggs and protozoa using zinc-sulphate faecal flotation; and

Seven key aims are addressed in the following chapters:

i) To assemble the most complete compilation of the health and diseases knowledge of the quokka, and update such knowledge with new data (Chapter 2).

ii) To determine the presence or absence of *Salmonella* spp., serovar richness and prevalence, as well as the impact of *Salmonella* spp. infection on the animals through physical examination, haematology and blood chemistry parameters (Chapter 4);
iii) To determine the presence or absence of *Cryptococcus* spp. in the nasal cavity of quokkas, the species present and their prevalence; as well as the impact of *C. neoformans* var. *grubii* infection on the animals through physical examination findings, haematology and blood chemistry parameters (Chapter 5);
iv) To determine the presence or absence of herpesvirus Deoxyribonucleic Acid (DNA), identify any herpesvirus detected, establish their prevalence, and assess the impact of herpesvirus infection on the animals through physical examination, haematology and blood chemistry parameters (Chapter 6);
v) To formulate haematology and blood chemistry reference intervals for apparently healthy animals, and determine the presence or absence of intraerythrocytic parasites and trypanosome DNA, identify the amplified DNA to genus and establish its prevalence, and the presence of microfilariae in peripheral blood (Chapter 7);
vi) To determine vitamin E concentrations in plasma for wild quokkas, and compare these values with those of captive animals (Chapter 7); and
vii) To formulate population health management procedures to facilitate on-going management of wild and captive populations (Chapter 8).

1.3 Chapter organisation

This thesis describes an investigation into the health and diseases of free-ranging quokkas from Rottnest Island and three subpopulations on the mainland.

- Chapter 1 reviews the impact of disease in free-ranging wildlife populations, and the importance of disease surveillance and health monitoring as the only tools that allow conservation bodies and decision makers to adequately manage and respond to disease in wild populations. This first chapter also contains the aims and chapter organisation to this thesis.

- Chapter 2 was written in a manuscript format and introduces the study animal. It covers multiple subjects ranging from behaviour to taxonomy as well as the most relevant aspects of its conservation status (e.g. distribution, threats, and captive populations). This chapter contains a comprehensive compilation of disease processes and agents of disease detected in the quokka. These data were compiled

from peer review literature and data available at conservation and wildlife veterinary databases.

- Chapter 3 describes general materials and methods, including trapping protocols and locations, field protocols for anaesthesia, physical examination, sample collection and handling, and general protocols for laboratory procedures such as haematology, blood chemistry, and faecal flotations.

The experimental chapters (Chapter 4 to 7) then follow. These four chapters were written in the format of manuscripts for submission to scientific journals. These chapters provide an introduction to the given organism, their significance in wildlife and the reasons why they were studied in the quokka. For each chapter, this is then followed by a description of the study design and the materials and methods implemented. Lastly, there is a results section for each chapter that is followed by a critical discussion of the findings pertaining to that given organism and a conclusive statement. Additional data not included in these experimental chapters is described in full in the Appendices.

- Chapter 4: *Salmonella* spp. was of interest to this project for three main reasons. First, the long-standing view that *Salmonella* infections in the Rottnest Island quokka were not associated with disease needed to be examined using thorough clinical methods. Second, there has been a temporal gap of more than 20 years in the surveillance of this infectious agent on Rottnest Island. This needed to be updated. And third, that the prevalence of *Salmonella* on the mainland quokka needed to be re-examined and compared to historical data and against that of the Rottnest Island population. This chapter investigates the prevalence and distribution of *Salmonella* spp., characterises the isolates recovered and assesses the implications of the infection on the health of quokkas.

- Chapter 5: *Cryptococcus* organisms, particularly *C. neoformans* var. grubii, *C. neoformans* var. *neoformans*, and *C. gattii* were examined in this project for two main reasons. First, the absolute absence of data concerning these highly pathogenic yeasts in the quokka, organisms that are known to cause debilitating disease (sometimes fatal) in wildlife and humans, needed to be addressed. Second, given that cryptococcal organisms and cryptococcosis, have been diagnosed with relative frequency in captive populations of Rottnest Island individuals, data to understand the possible relation between these yeasts and free-ranging quokkas is epidemiologically important. This chapter investigates the prevalence and

distribution of *Cryptococcus* spp. and other yeasts, characterises the isolates recovered, and assesses the implications of the infection on the health of quokkas.

- Chapter 6: Herpesviruses were of interest to this project for various reasons. First, given the fatal case of a quokka that succumbed to MaHV-2 infection in a captive colony in the 1980s (Wilks, Kefford, & Callinan 1981), it was important to obtain evidence of previous exposure to MaHV-2 in free-ranging animals, and to investigate whether the quokka was the natural host or not. Second, herpesviruses are known to persist in the host as a latent infection and cause clinical disease under stress (usually fatal disease in other species if shedding of the virus occurs) and so determining the presence or absence of a herpesvirus in the quokka was considered to be epidemiologically significant. This chapter investigates the prevalence and distribution of herpesviruses in quokkas, characterises the detected herpesvirus, and assesses the implications of the infection on the health of quokkas.

- Chapter 7: Although focused primarily on the establishment of haematology and blood chemistry reference intervals for Rottnest Island and mainland quokkas, and exploring the differences between these two in the context of disease, it also studied haemoparasites (e.g. *Babesia* sp., *Theileria* sp. and microfilariae), and vitamin E in plasma (of great interest to institutions with captive populations). This chapter reviews the importance of these ancillary tests in the assessment of health in wildlife, the need for reference intervals, and the dearth of data in the quokka. Samples were collected and analysed so that reference intervals could be established.

- Chapter 8 is a general discussion of chapters 4-7. An attempt to analyse the cofounding effects of multiple infections on the haematology and blood chemistry of quokkas on Rottnest Island and the mainland is included. Overall, this chapter attempts to place the findings of the experimental work in the context of health and management of the quokka.

2. Diseases and pathogens reported in the quokka, *Setonix brachyurus* (Quoy & Gaimard 1830) (Marsupialia: Macropodidae) with notes on its ecology, status and threats: a review

STATEMENT OF AUTHOR CONTRIBUTION

P. Martinez-Perez: designed the general scope and structure of the chapter, initiated the research, performed the collection and collation of the literature, and wrote the chapter.

P.A. Fleming: provided editorial comments to versions of the chapter from draft to final version.

T.H. Hyndman: provided editorial comments to versions of the chapter from draft to final version.

2.1 Abstract

Disease, as other ecological factors (e.g. fire regimes, climate, drought, predators) plays an important and dynamic role in the persistence of wildlife populations. Previous studies have shown that disease can push species to drastic declines or even extinction (e.g. facial tumour disease in Tasmanian devils, white nose syndrome in bats, and chytridiomycosis in amphibians). Although disease has not been linked to the current decline of the species, there are anecdotal records of numerous events of disease in the mainland quokka. Because of the risk of potential catastrophic effects of disease on the conservation status of the species, preventive management and mitigation actions of such impacts required not just active surveillance but a better understanding of infectious agents and disease processes in the species. Disease and agents of disease have been reported in the quokka since as early as 1914, however, the information is too scattered to be useful, and there is no consensus on how much is known of the species. This review offers a comprehensive description of diseases and agents of disease in the quokka since as early as 1914, attempts to correct misleading data, describes the species, updates some conservation status data, and proposes some possible avenues of research.

2.2 Introduction

The quokka, *Setonix brachyurus* (Quoy & Gaimard 1830), has been the object of research on a variety of topics since the early 1900s with studies and reports concerning the health and diseases of this species dating back to at least 1914. Microbiology and parasitology are the two most commonly described subjects but reports concerning nutrition and neoplastic disorders also exist. Physiology and disease studies have involved both free-ranging and captive individuals; for example, hormonal secretion, lactation, immunology and organ development have been investigated (Ashman, Holmes, & Keast 1977 ; Chauvet *et al.* 1983 ; Fleming, Harman, & Beazley 1996 ; Jordan & Morgan 1968 ; Makanya *et al.* 2001). Interestingly, the species has also played an important role as an animal model to study concepts in human psychology, such as detour behaviour and transverse patterning (Bonney & Wynne 2004 ; Wynne & Leguet 2004).

Ecological studies have examined the distribution of the species and threatening processes, with particular emphasis on populations from Rottnest Island and the northern jarrah forest (i.e. mainland) (Dunnet 1962, 1963 ; Hayward 2002 ; Hayward 2005, 2008 ; Hayward *et al.* 2004 ; Hayward *et al.* 2007 ; Hayward *et al.* 2003). Some of the threats affecting the species include predation by feral animals, habitat destruction, and climate change (de Tores *et al.* 2007 ; Department of Environment and Conservation 2013). Although the Rottnest Island subpopulation appears reasonably stable, the species is under a continuing decline in extent of occurrence and area of occupancy (de Tores *et al.* 2008 ; de Tores *et al.* 2007). As a result, the quokka is listed as "fauna which is rare or likely to become extinct" under the Western Australian Wildlife Conservation Act 1950 Section 14(2) (ba); and as "threatened fauna" subcategory "vulnerable" under the Commonwealth of Australia's Environment Protection and Biodiversity Conservation Act 1999. Similarly, the species holds a conservation status classification of "vulnerable" according to the International Union for Conservation of Nature (IUCN) (de Tores *et al.* 2008).

Disease has not been linked to the ongoing decline of the quokka, however, there are anecdotal records suggesting that the species may have had several epidemics of disease since the 1900s, particularly mainland groups (Abbott 2008 ; Perry 1973 ; White 1952). Although reasonably stable, even the Rottnest Island subpopulation is at risk of stochastic events, such as fire which destroys habitat for these animals, such as has been witnessed in the past (Rippey & Hobbs 2003). Likewise, an infectious disease outbreak could present a catastrophic event that could result in a severe crash of the population, or

possibly its extinction. As an example in other species with island populations, the Devil Facial Tumour Disease (DFTD) represents a serious threat to the Tasmanian devil (*Sarcophilus harrisii*) (Hawkins *et al.* 2006 ; McAloose & Newton 2009 ; McCallum 2008). The species was abundant and spread throughout Tasmania before DFTD appeared in 1996, with an estimated population size of ~150,000 individuals (Hawkins *et al.* 2006). Positive Tasmanian devil subpopulations have declined by up to 95% and data does not indicate a decrease in incidence, prevalence or declined of this disease (Philips & Driessen 2008).

Due to the variety of topics studied on the quokka, the volume of work performed thus far, the ex-situ programs that are currently being carried out at different institutions across Australia (see section 2.3.2), a new recently-approved recovery plan for the species (Department of Environment and Conservation 2013), and an action plan to conserve threatened Australian macropods (Roache 2011), it is now an opportune time to compile and review the conservation status of the quokka, together with the diseases and agents of disease that have been reported on the species.

2.3 Description

The species (Figure 2-1) was first described in 1658 by the Dutch explorer Samuel Volckersen after landing on Rottnest Island. In 1696, Willem De Vlamingh described the species as 'a kind of rat as big as a common cat', and named Rottnest (or "rat's nest") Island after it (Croft 1991). The quokka is a small, diprotodont [1] wallaby marsupial, with thick and coarse grey to brown fur. It has a naked snout, short ears and a close-haired tail. Males weigh 2.7 – 4.2 kg, have an average head-body length of 487 mm (435 – 540 mm), and an average tail length of 289 mm (260-310 mm) and females weigh 2.5 – 3.5 kg, have a length ranging from 400 – 500 mm, and an average tail length of 265 mm (245 to 285 mm) (Kitchener 1995). The quokka can store fat in their tails as a mechanism to handle with seasonal food availability (Sinclair 1998). The Noongar people of the south-west of Western Australia (WA) know the quokka by a variety of names including '*kwoka'*, '*bangop*' (Abbott 2001), and '*Ban-gup*', *Bangeup*', and '*Quak-a*' (Gould 1863 ; Shortridge 1909).

Today, it is generally accepted that the quokka belongs to the family Macropodidae given its skull structure, dentition and tail. However, the difference in chromosomes morphology and fundamental number (i.e. number of chromosome arms) when compared

[1] from the Greek *diprotos*, meaning 'two front' and *odontos* meaning 'teeth'

to other members of the family Macropodidae (Sharman 1961), place this species outside the typical wallabies in its own genus: *Setonix*. Nonetheless, the species has had an interesting history in terms of its taxonomic classification. Lydekker (1894) placed the quokka in the genus *Macropus* together with the parma wallaby (*Macropus parma*), and the tammar wallaby (*Macropus eugenii*) among others. This genus assignation was supported by Bensley (1903) on the basis of the terrestrial character of the foot and the loss of the canines, despite the strong resemblance of its pre-molars and molars with those of the genus *Dorcopsis* and tree kangaroos of the genus *Dendrolagus*. Subsequent classification by Jones (1924) recognised the quokka as a brachydont section of the Macropodinae subfamily, given the shared characteristics in pre-molar and molar teeth with other members of this subfamily (e.g. *Dorcopsis* and *Dendrolagus*), but from which it differs by the absence of canines and having a short tail. Further changes to its nomenclature were carried out by (Tate 1948 ; Throughton 1941). After studying the karyotype of the quokka, Sharman (1954) proposed that the species was most probably related to the genus *Thylogale*, based on the similarity of its number of chromosomes with the number of chromosomes of the Tasmanian pademelon (*Thylogale billardierii*). However, later it was found that although the number of chromosomes was the same, their morphology was different and the female quokka had more chromosome arms than usual (between 36 and 40) (Sharman 1961). In (1957), Ride presented a strong case that would assign the quokka to the group of rat-kangaroos, however, dental and cranial features made him conclude that the species should remain within the Macropodinae subfamily, although it was also recognised that more comparative studies were needed. In any case, today's literature does not clearly state when and who reinstated the genus *Setonix*. Despite the general view that the quokka belongs to the Macropodidae family, there are still some modern documents assigning the species to the Potoroidae family e.g. The Action Plan for Threatened Australian Macropods 2011-2021 (Roache 2011). However, this is contrary with accepted taxonomy of these animals because the Potoroidae have well-developed upper canines that are not present in *S. brachyurus* (Hume *et al.* 1989 ; Seebeck & Rose 1989).

Although there are a reasonable number of studies on the taxonomy of *S. brachyurus*, and that for the most part the general hypothesis is that the species belongs to the family Macropodidae, it is important to acknowledge that there is a lack of genetic resolution to the classification of *S. brachyurus*. An example of how genetic studies supersede old classification techniques such as those on which the genus *Setonix* and the species *brachyurus* (for the most part morphometric measurements and phenotype differences) stand, is that of *Pseudonovibos spiralis*. *P. spiralis* was identified as a new species of bovid

by Peter and Feiler (1994), on the basis of horns (i.e. phenotype and morphometric features) found in Cambodia. For this reason, the "species" was quickly listed by the IUCN as endangered. However, later molecular studies targeting the 12S rRNA gene, the mitochondrial cytochrome b gene, and the nuclear lactoferrin gene, determined that the horns were fake (i.e. manually modified), and belonged to other species (Brandt *et al.* 2001). Consequently, *P. spiralis* was removed from the IUCN and does not appear in the Catalogue of Life (http://www.catalogueoflife.org/).

Figure 2-1 Female quokka (*Setonix brachyurus*)

2.3.1 *Geographical range*

Based on published and unpublished accounts, de Tores et al. (2007) suggests that the quokka once occurred over a total area of ~44,300 km^2, from Jurien Bay (~220 km north of Perth) to Albany and as far east as Hunter River, including offshore islands (e.g. Rottnest Island ~20 km west of Perth, and Bald Island, east of Albany which is ~400 km SE of Perth). Shortridge (1909) indicated that the range of the species extended from Moore River (~100 km north of Perth) to east of Esperance (~720 km SE of Perth), including Twin Peak Island (off the coast ~80 km east of Esperance) and other small islands, but was mostly restricted to a habitat of coastal thickets and swamps. The quokka has suffered a substantial range contraction subsequent to European settlement, such that

by 1992, the total extent of occurrence is estimated as ~17,800 km[2] (Department of Environment and Conservation 2013).

It has been estimated that the Bald Island subpopulation separated from the mainland approximately 10,000 years before present (Storr 1965), while Rottnest Island separated approximately 7,000 years before present (Glenister, Hassell, & Kneebone 1959). These populations have been relatively stable, which may reflect their safety from introduced predators. On the mainland, today the species is characterised by subpopulations [2] that go as far north as Churchman Forest Block (~10 km SE of Perth), and southwards along the 1,000 mm annual rainfall isohyet, including forested areas around the towns of Collie (~200 km south of Perth), Manjimup (~294 km south of Perth), Walpole (~411 km SSE of Perth) and around the city of Albany (~418 km SE of Perth). The most eastern boundary of the distribution today is near Waychinicup National Park, approximately 62 km east of Albany (de Tores *et al.* 2007).

2.3.2 *Populations and status*

Population estimates suggest that there are fewer than 18,000 animals across all subpopulations (Table 2-1), which are believed to be isolated. However, considering the elusive nature of this marsupial on the mainland of WA, and that counting every single individual of *S. brachyurus* is not possible, these population estimates could be much less than what it is believed. This would be particularly the case for *S. brachyurus* on the mainland.

Table 2-1 Subpopulation estimates [a] for various locations where quokkas occur in Western Australia.

Locations	Number of animals
Rottnest Island	4,000-8,000 [a], 8,000-12,000 [b]
Bald Island	500-2,000 [a], 600-1,000 [b]
Northern Jarrah forest	150 [a], <110 [b]
Central Jarrah forest	<100 [b]
Southern forests	2,000-5,000 [a], >700 [b]
Stirling Range	>50 [b]
South Coast	1,200-2,000 [a], >250 [b]

[a] IUCN data (de Tores *et al.* 2008)
[b] Quokka Recovery Plan data (Department of Environment and Conservation 2013)

Hayward et al. (2003) determined that the age distribution of mainland quokka (northern jarrah forest subpopulation) were 50% adults, 25% juveniles and 25% pouch young. A similar age distribution was reported by Dunnet (1963) for the Rottnest Island population. However, the age distribution of quokkas on the northern jarrah forests may have

[2] a geographically or otherwise distinct group between which there is little demographic or genetic exchange (IUCN 2015).

changed since the last assessment was done in the early 2000s, as Dundas et al. (unpublished data) obtained a larger ratio of adult to immature animals (7.4:1) in comparison to Hayward et al. (2:1) (2003), while surveying the northern jarrah forest subpopulations. The population composition of the southern populations is not known. Localised population collapse was suggested for the northern jarrah forest subpopulation (Hayward *et al.* 2003). However, it is believed that numbers have been rising over time and today there seems to be a reasonable number of animals on the northern jarrah forest (S. Dundas, *pers. comm.* 2013). The land usage in which these seven subpopulations are found includes state forests (northern jarrah forest, central jarrah forest and southern forest subpopulations), Class 'A' reserve (Rottnest Island subpopulation), nature reserves (south coast and Bald Island subpopulations), and national parks (Stirling Range) (Department of Environment and Conservation 2013). In Australia, there are approximately 108 individuals of *S. brachyurus* in captivity, of which approximately 86 are part of collections in other states different than WA (Table 2-2).

Table 2-2 Number of individuals of *S. brachyurus* in captive collections in Australia and their responsible organisations. WA= Western Australia, NSW= New South Wales, SA= South Australia, VIC= Victoria, NA= not available.

Project	Location	Current no. of animals (Department of Environment 2013b)	This study	Females	Males	Unknown sex	Notes
Broome Wildlife Park	WA	3	NA	-	-	-	no record of any project by that name [a]
Caversham Wildlife Park	WA	16	7 [b]	3	4	-	all from Rottnest Island
Cohunu Wildlife Park	WA	4	None [c]	-	-	-	fate of the animals is unknown
Peel Zoo (Mandurah)	WA	NA	2 [b]	2		-	-
Perth Zoo (Perth)	WA	3 (mainland)	13 [d]	10	3	-	all from Rottnest Island
Quindalup Wildlife Park (Dunsborough, Western Australia)	WA	3	None	-	-	-	closed down in mid 2012 [e], fate of the animals is unknown
University of Western Australia-Animal Care Services at Shenton Park	WA	24 (Rottnest Island)	None	-	-	-	this facility has not held quokkas since August 2012 [f]
Adelaide Zoo	SA	NA	18 [b]	13	5	-	all from Rottnest Island
Ballarat Wildlife Park	VIC	NA	15 [b]	9	4	2	all from Rottnest Island
Gorge Wildlife Park	SA	NA	11 [b]	4	6	1	all from Rottnest Island
Halls Gap Zoo	VIC	NA	3 [b]	2	1	-	all from Rottnest Island
Healesville Sanctuary	VIC	NA	1 [b]	-	1	-	all from Rottnest Island
Melbourne Zoo	VIC	NA	14 [b]	8	5	1	all from Rottnest Island
Taronga Western Plains Zoo	NSW	NA	5 [b]	3	1	1	all from Rottnest Island
Taronga Zoo	NSW	NA	16 [b]	8	7	1	all from Rottnest Island
WILD LIFE Sydney Zoo	NSW	NA	3 [b]	-	3	-	all from Rottnest Island

[a] Source: Broome's visitor centre indicated that there is no record of a wildlife park by that name, and further information was not available
[b] Source: Zoo and Aquarium Association online census and plan, accessed 16th May, 2014
[c] Source: electronic communication via the general 'contact us' e-mail at the Cohunu Wildlife Park website (21st May, 2014)
[d] Source: Rebecca Vaughan *pers. comm.* 2014 (as of May 2014)
[e] Source: Robert Harris *pers. comm.* 2014
[f] Source: Simone Chapple *pers. comm.* 2014

According to Sinclair (1998), there are significant differences in the morphology of the different geographical populations of *S. brachyurus*. Generally, animals on Rottnest Island have larger dimensions (i.e. pes and tail length, as well as tail width but not necessarily heavier) than those on the mainland and on Bald Island, with the latter being the smallest of all. In 2001, the mitochondrial and allozyme analyses undertaken by Sinclair showed a genetically diverse mainland population whereas island populations had low levels of genetic variation. Alacs et al. (2011) further confirmed this, reporting a high degree of genetic differentiation between the northern jarrah forest subpopulation and Rottnest Island individuals. Other comparisons between other geographical subpopulations have not been undertaken.

2.3.3 ***Reproduction***

Reproductive parameters have been studied the most on Rottnest Island animals in captive conditions, with little work done on Bald Island and mainland specimens. The species is polyoestrous (Sharman 1955a) and all populations (Bald Island, Rottnest Island and mainland) have clearly defined breeding patterns. Though the species is polyoestrous, free-ranging Rottnest Island females usually give birth once a year during January (southern hemisphere summer). However, births have been documented to occasionally occur in August (during Winter) (Shield 1964). According to Shield (1968), *S. brachyurus* on Bald Island also breed seasonally. Breeding starts in February (Summer) and births happen in March or April (Autumn), and occasionally on November. Around 18 months of age, a seasonal anoestrous of six months has been documented in captive female joeys that were originally from Rottnest Island. This means females do not have their first joey until they have reached at least 24 months of age (Shield 1964). Though not thoroughly studied, it is believed that Bald Island females do not have an anoestrous period (Shield 1968). It has been demonstrated that captive conditions modify breeding patterns, with females from Rottnest Island losing their anoestrous period after two years in captivity, and after that, breed all year round (Sharman 1955a ; Shield 1964), and both mainland and Rottnest Island females may start breeding as early as eight or nine months of age (Shield 1968). On the mainland, it has been generally known that quokkas have no breeding season and births occur throughout the year (Hayward *et al.* 2003 ; Shield 1964). However, a recent study by Dundas et al. (unpublished data) suggests that quokkas on the northern jarrah forest have started to breed seasonally, with the highest number of births occurring during March and April. The authors indicate that this might be a nutritional driven adaptational change, possibly in response to the negative effects of climate change on the vegetation. According to Sharman (1955a), the Rottnest Island female (in captivity)

reaches reproductive life around 18 months of age; whereas males reach sexual maturity around 389 days (Shield 1968). *Setonix brachyurus* has a gestation length of about 25 to 27 days (Sharman 1955b ; Shield 1968) with delayed blastocysts completing development in one to two days less than a full gestation (Shield 1968). Shortly after birth (usually one day) the dam enters oestrus and ovulation occurs (Sharman 1955b). If fertilisation happens post-partum, the resulting embryo remains unimplanted (diapause) while the pouch is occupied by a suckling pinky. These blastocysts, suspended in development, can remain in this stage for up to five months (Sharman 1955b). A typical pregnancy involves only one joey, however, pregnancies with two joeys have been documented for Rottnest Island animals (Hayward *et al.* 2003). Joeys emerge from the pouch between 175 and 195 days (Department of Environment and Conservation 2013) and weaning occurs four to six months later. There are no studies on the reproductive behaviour of the species.

2.3.4 *Life span*

So far the exact life span of the species has not been confirmed and the literature reports different numbers. Crandall (1964) recorded that specimens lived for six to ten years in captivity. Holsworth (1964) reports observing ten years later, a Rottnest Island male that was first tagged and released at the age of 270 days. Weigl (2005) reports 13.8 years in captivity; while the longest life span today for *S. brachyurus* is reported by Fleming et al. (1996) and puts the oldest animals at 15 years old (in captivity).

2.3.5 *Behaviour*

Natural behaviour of the quokka seems to be poorly documented, particularly for mainland animals, due to their relatively small size, nocturnal behaviour, and the thick understory they live in. Available data refers principally to Rottnest Island animals or animals held in a captive environment.

Setonix brachyurus is not a burrowing species and does not make nests; instead, it creates shelters and runways under bushes and scrub (Dunnet 1962). Although a ground-dwelling species, quokkas have been reported to climb onto bushes about ~1.5 m from the ground for feeding (Dunnet 1962). The species is mainly nocturnal; however, individuals on Rottnest Island can be seen active during the day. While feeding, animals are often solitary except for dams that have joeys at foot. It has been suggested that captive individuals from Rottnest Island have a social structure characterised by a linear hierarchy and territoriality (Packer 1969).

2.3.6 *Diet*

The primarily herbivorous diet of the quokka varies with geographical location. Storr (1964) reported that the diet on Rottnest Island varies according to the season and location, with succulent species (e.g. *Carpobrotus aequilaterus* or angular pigface) being the main component, and grasses and shrubs in a smaller proportion. A seasonal (autumn) deficit in dietary nitrogen and water throughout the year on Rottnest Island was also determined. Although not irrefutable, recent data from Poole et al. (2014), revealed a possible shift in the diet habits of the Rottnest Island quokka, in which *Guichenotia ledifolia* was the most abundant plant present in faecal samples, whereas *Carpobrotus aequilaterus* was found to be the primary food source in Storr (1964) studies. In addition, animals on Rottnest Island have been seen supplementing their diet with fungi (Chapman 1999) and snails (Erickson 1951). On the mainland, Hayward (2005) concluded that northern jarrah forest animals prefer leaves and stems, with shrubs from the *Thomasia* genus being the most common component of the diet. Other plants consumed at these mainland sites included *Bossiaea aquifolium* (water bush), and *Mirbelia dilatata* (holly-leaved Mirbelia). Data on the diet of the southern populations was not available at the time this was written.

2.3.7 *Habitat*

In the northern-most forests of their natural range, the species prefers (but are not limited to) dense vegetation around swamps and watercourses, characterised by both the presence of the swamp peppermint (*Taxandria linearifolia*) and a mosaic structure of unburnt and burnt vegetation (de Tores *et al.* 2007). Subpopulations in the southern forests inhabit a wider range of habitats such as dense streamside beds, *Eucalyptus diversicolor* (karri) regrowth, *Eucalyptus jacksonii* (red tingle) and *Eucalyptus guilfoylei* (yellow tingle) forests, as well as areas with *Eucalyptus marginata* (jarrah), *Corymbia calophylla* (marri) and spreading sword-sedge understory (de Tores *et al.* 2007 ; Department of Environment and Conservation 2013). On Rottnest Island, habitat has drastically changed since European settlement. Initially, the presence of native pines [e.g. *Callitris preissii* (Rottnest Island pine or cypress)], tea-trees [e.g. *Melaleuca lanceolatta* (Rottnest tea tree)], and *Acacias* spp. were abundant (Dunnet 1962 ; Stevenson 2011). Today, open areas in Rottnest Island are dominated by *Acanthocarpus preissii* (prickle lily) and *Stipa variabilis* (variable speargrass) (Dunnet 1962); urban areas of the island are characterised by exotic species (e.g. *Eucalyptus platypus*, and *Araucaria heterophylla*) that were established for aesthetic reasons (Stevenson 2011). In comparison with the

mainland, Rottnest Island does not have streams of fresh water and water availability at lakes (not on the West End) is limited due to high salinity (Hodgkin 1959).

2.3.8 *Threats and decline*

The quokka has suffered a marked range contraction since European settlement (Department of Environment and Conservation 2013). A marked decline of the quokka was recorded during the 1930s (White 1952), with hunting playing an important role as the species was declared a pest in 1933 (Department of Environment and Conservation 2013). Threatening processes to the survivorship of *S. brachyurus* and patterns of decline have been reviewed by de Tores et al. (2007) and in 2013 by the Quokka Recovery Plan (Department of Environment and Conservation 2013). The decline of Western Australia's terrestrial mammalian fauna within the critical weight range (CWR; 35 g - 5.5 kg) was primarily attributed by Burbidge & McKenzie (1989) to environmental changes, including predation and competition by introduced eutherian animals such as the domestic cat (*Felis silvestris catus*), the dingo (*Canis lupus dingo*) and the European red fox (*Vulpes vulpes*), as well as altered fire regimes, reduction of vegetation cover, climatic changes and hunting. Some anecdotal reports suggest that disease may have also been involved in the decline of the quokka on WA (Abbott 2006, 2008). However, a revision of the CWR by Johnson and Isaac (2009) concluded that terrestrial species in low rainfall areas are at a higher risk of extinction. This may indicate that the quokka, that requires high rainfall conditions (in excess of 700 mm, de Tores *et al.* 2007), could have been affected to a much lesser extent than other terrestrial species in the CWR. However, this high rainfall requirement may reflect their reliance on vegetation cover, which in turn will make the species highly susceptible to vegetation changes.

Among the introduced predators, the European red fox is generally accepted as a significant predator of medium size terrestrial mammals (Burbidge & McKenzie 1989 ; Johnson, Burbidge, & McKenzie 1989 ; Risbey *et al.* 2000 ; Short & Smith 1994) and predation pressure by this species has been suggested to be the most important factor acting in the decline of the quokka in the 1930s (de Tores *et al.* 2007 ; Department of Environment and Conservation 2013 ; White 1952). The quokka Recovery Plan 2013, indicates that fox control has been linked with an increase in trap success of *S. brachyurus* at Mount Manypeaks and that overall, there is a good amount of anecdotal evidence that points at an increase in abundance of *S. brachyurus*. Quoting de Tores et al. (2007), 'it seems very likely foxes were responsible for the initial decline of the quokka on the mainland and have contributed to its continued decline'. Interactions with other species such as the feral cat, the dingo and the feral pig (*Sus scrofa*), may have played a role in the

decline, but to a much lesser extent. Nonetheless, these other species are still considered a current threat (de Tores *et al.* 2007 ; Department of Environment and Conservation 2013). However, de Tores et al. (2007) believed that the use of runways and the thick vegetation of their habitat may have made *S. brachyurus* less vulnerable to predation. Although this is not proven, other authors have suggested that particularly difficult habitats play a protective role for other prey species (Burbidge & McKenzie 1989 ; Kinnear, Onus, & Bromilow 1988). Nonetheless, historical and current range contractions indicate not only that the European red fox was probably the most important factor, but also that there are other factors that have participated in the decline of the species (Department of Environment and Conservation 2013).

Climatic changes alone are still not considered sufficient to be the sole reason responsible for the decline of *S. brachyurus* since the 1930s, however, changes in rainfall patterns and increase of global temperatures are still considered a threat to the survivorship of the species (Department of Environment and Conservation 2013 ; Shortridge 1909). The risk of extinction and redistribution of fauna and flora in Australia and around the globe by changes in the Earth's climate has been explored by various authors (Brereton, Bennett, & Mansergh 1995 ; Malcolm *et al.* 2006 ; Peterson *et al.* 2002 ; Thomas *et al.* 2004 ; Williams, Bolitho, & Fox 2003) and *S. brachyurus* has been included in these studies (Gibson *et al.* 2010). For instance, Gasner et al. (2010) found that nearly half of the bird species in montane rain forest habitats from Costa Rica, are expected to decline in the next century. Malcolm et al. (2006) identified the southwest of Australia as one of the seven especially vulnerable 'biodiversity hotspots' in a climate scenario containing double the concentration of CO_2, with more than 2,000 plant species extinctions happening. Thomas et al. (2004) projected a 15-37% systematic decline of the species by 2050 under a global temperature increase of 1.8-2.0 ˚C and a global CO_2 concentration increase of 500-550 p.p.m.v (parts per million by volume), coupled with habitat destruction. According to Gibson et al. (2010), under the predicted increased aridity of south-western Australia as a result of a 4 ˚C increase in global temperature, *S. brachyurus* may lose all of its current distribution range by 2070. According to the Scripps CO_2 Monitoring Program (Scripps Institution of Oceanography, La Jolla, California), as of December 2013, the concentration of CO_2 in Earth's atmosphere was 396.73 p.p.m. This was subsequently reviewed in June 2015 and the atmospheric CO_2 had increased to 403.70 p.p.m. (CO2Now 2013).

Fire regimes post European settlement may have been implicated in the early decline of *S. brachyurus* (de Tores *et al.* 2007 ; Hayward 2002). Before European settlement, Aboriginal people are thought to have used fire with low intensity and a given frequency

(i.e. 2 - 3 years for high rainfall areas, and 2 - 5 years for low rainfall areas) which is believed to have led to vegetation characterised by long unburnt areas and the presence of recently burnt areas (Burrows, Ward, & Robinson 1995). More recently, Hayward (2002) concluded that this vegetation structure with a mosaic of unburnt (source of protection) and freshly burnt (source of food) areas was preferred habitat for *S. brachyurus*. Today, the quokka Recovery Plan 2013 considers this vegetation structure as 'critical habitat' to the survival of the species. In the southern forests, occupancy of *S. brachyurus* is best predicted by habitat patchiness, low density of woody debris and a complex vegetation structure of minimum three layers (Bain, Wayne, & Bencini 2015), with a varied fire-age mosaic structure (Bain et al. in prep. (a), in Department of Environment and Conservation 2013). However, fire regimes that changed after European settlement (de Tores *et al.* 2007) may have exposed *S. brachyurus* to higher mortality rates due to higher predation pressure from the European red fox (Hayward *et al.* 2007). Conversely, the quokka Recovery Plan 2013 indicates that there is not sufficient evidence to infer that lack of fire has a negative impact on the survivorship of wild populations of *S. brachyurus*. However, not using fire can negatively impact quokka habitat by means of midstorey aging, collapse and accumulation of bio-fuel (Bain et al. in prep. (a), in Department of Environment and Conservation 2013). A reduction of the interval between fires, increased fire intensity and faster fire spread has been predicted (Cary 2002 ; Williams, Karoly, & Tapper 2001). Modelling suggests that by 2070, southwest WA could potentially experience 80% more drought-months if current climate trends continue (Department of Environment 2013a). Coupled with increasing global temperatures, this increasing aridity could result in a longer bush fire season together with fires of greater scale that threatens *S. brachyurus* habitat and hence their survivorship.

Most of the habitats used by *S. brachyurus* in the southwest of WA have been cleared, are now fragmented, and are easily invaded by feral species, primarily due to urban development. About 75% of the wetlands (e.g. swamps) on the Swan Coastal Plain (that historically supported *S. brachyurus*) have been drained, filled or modified (Gole 2006). Harvesting of ground and surface water will increase with the increasing urbanisation of the Swan Coastal Plain, and areas that currently sustain *S. brachyurus* will probably reduce in size.

Threatening processes on island subpopulations have a greater degree of impact on the survivorship of the species. Oceanic islands are naturally restricted habitats, in the sense of species living in them are surrounded by inhabitable conditions, and this is particularly the case for ground dwelling species, such as *S. brachyurus*. In theory, the isolated

populations of *S. brachyurus* on Rottnest Island and Bald Island, have a risk of extinction due to various factors, including: the inability to disperse and the impossibility of recolonisation (Diamond 1975), as well as loss of genetic variation by inbreeding depression (Caughley & Gunn 1996). Avoiding island-scaled threatening changes in their environment by naturally dispersing to 'safer' areas is not possible, and there are no 'source' populations that could naturally recolonise the island in the case of a serious demographic accident. Additionally, the biogeography of these islands seems to lack features that could act as physical barriers either against environmental events (e.g. flooding, strong winds and fires) or infectious diseases (i.e. depending on the mechanism of transmission). Consequently, in the context of infectious diseases, a small outbreak could potentially evolve into one of island scale magnitude. The devil facial tumour disease that affects the Tasmanian Devil (*Sarcophilus harrisii*) may be an example of this. This infectious cancer that causes 100% mortality in infected animals, is transmitted from animal to animal by allografts (i.e. direct contact, particularly biting) (Pearse & Swift 2006 ; Pye, Woods, & Kreiss 2015). With an original estimated population of 150,000 animals before the appearance of the disease (i.e. 1996), by 2008 the population had already declined to approximately 60%, and studies suggest that DFTD may be present throughout the entire range of the devil by 2018 (McCallum *et al.* 2007). According to Pye et al. (2015), cases of DFTD have been recorded in more than 80% of the Tasmanian devil range. This rate of transmission suggests that there is nothing in the biogeography of the Tasmanian devil range that creates a barrier against susceptible hosts getting in contact with infected hosts or vice versa. Furthermore, the disease has been associated with a loss of major histocompatibility complex diversity in the species, possibly as a result of inbreeding depression (Siddle *et al.* 2007). Similarly, poor genetic diversity has already been reported for the Rottnest Island quokka, with inbreeding depression being a possible cause (Alacs *et al.* 2011 ; Sinclair 2001).

According to the study by Purvis et al. (2000), the most important predictors of extinction risk are small geographic range and island endemicity. Likewise, oceanic islands have been recognised as biogeographical areas with a high *latent extinction risk* [3] based on the dominance of endemic species and the recognised role of small geographic range as a predictor of high extinction risk (Cardillo *et al.* 2006). Without a doubt, Rottnest Island and Bald Island fulfil two of the characteristics required to be classified as having populations with a high extinction risk. Additionally, Rottnest Island and Bald Island biogeography lacks features that could barrier against animal dispersion, therefore favouring animal to animal transmission of an infectious agent. Strictly speaking, although

[3] defined as the difference or discrepancy between a species' current extinction risk and that predicted from models on the basis of biological traits (Cardillo *et al.* 2006).

being reported a stable subpopulation, quokkas on Rottnest Island – as well as those on Bald Island – seem to be at a greater risk of extinction than those on the mainland.

The role-played, if any, by diseases in the historical decline of *S. brachyurus* has been ruled out for the most part. This may largely be because the majority (if not all) of records of disease in quokka since the 1920s are anecdotal accounts. A drastic decline occurred in between 1938 and 1944, with factors such as habitat loss and predation considered to have had a role in the decline, however, could not explain a widespread and abrupt reduction in numbers (Perry 1973). White (1952) states that bushmen considered that the decline of *S. brachyurus* in the 1930s was mainly the result of disease and that all other factors (e.g. habitat clearing, predation) were just supplementary to it. However, Burbidge and McKenzie (1989) indicated that extinctions or permanent declines in Western Australian mammalian fauna could not be explained solely on the bases of disease. Johnson et al. (1989) reinforces this view by arguing that it is highly unlikely that a disease process will selectively target species within the CWR, but does not entirely rule out the possibility by stating "we consequently regard exotic disease as a potential but unlikely cause of decline and extinction in macropods". Nevertheless, the anecdotal data documenting mass death events in *S. brachyurus* during the same period is numerous.

De Tores et al. (2007) retrieved state records of mass mortality in quokkas attributed to disease: three records from 1921 (near Manjimup ~300 S of Perth), and one record from 1933 (near Yallingup ~250 km SW of Perth). Similarly, Abbott (2006, 2008) presents a case that strongly suggests that disease played a significant role in the early decline of mammals on WA, particularly between 1880 and 1940. In the case of *S. brachyurus*, the anecdotal data points at accounts of disease in 1905 with quokkas being affected by 'some kind of fur disease that only about one out of six animals were fit to skin'. Records from 1901, 1920 and 1921 referred to animals being found dead in great numbers. Subsequently, anecdotal reports from 1931 (around Middlesex ~300 km south of Perth) describe 'quokkas moving in circles until they died'. Reports from the mid 1930s described animals considered to be sick, with 'eyes puffed up and discharge from ears and noses' (around Peerabeelup ~310 km south of Perth). Lastly, between 1934 and 1936, records describe animals in which the skin was 'easily pulled off and pus was present between the inner and outer layers of it, as well as in the nose'.

Detailed medical descriptions of the clinical signs observed in quokkas between 1905 and 1931 were not produced, thus it is difficult to exclude infectious disease as a potential aetiology responsible for the mortalities observed during this period. Some of these

anecdotal descriptions seem to resemble clinical signs found in disease conditions known today. For example, '*circling*' (i.e. moving in circles), an unspecific clinical sign typically present when there has been damage of the central nervous system (CNS), particularly the forebrain and/or the vestibular system (Thomson, Hann, & Johnson 2012). This damage is most commonly associated with direct insult to the CNS by infectious organisms (viral, bacterial, fungal, parasitic), space-occupying lesions (e.g. tumours and abscesses), toxicoses and trauma (although less common); as well as in animals with liver disease (e.g. hepatic encephalopathy) and other conditions (Metre & Mackay 2015 ; Nelson & Couto 2009). This clinical sign has been previously described in some native Australian marsupials such as the Gilbert's potoroo (*Potorous gilbertii*) and the long-nose potoroo (*Potorous tridactylus*) with severe multifocal granulomatous meningoencephalomyelitis due to infection with *Cryptococcus neoformans* and *Cryptococcus gattii* (Vaughan *et al.* 2007). Similarly, it has been reported in the common brushtail possum (*Trichosurus vulpecula*) with encephalitis due to infection with *Toxoplasma gondii* (Ladds 2009). Likewise, *circling* is present in domestic mammals affected by a variety of infectious diseases (Table 2-3).

Table 2-3 Some diseases in which *circling* has been documented in domestic mammal species.

Species	Disease	Reference
Cattle	Listeriosis, Trypanosomiasis, Theileriosis	(Metre & Mackay 2015)
Dogs and Cats	hepatic encephalopathy, neural larvae migrans and heartworm disease	(Ware 2009 ; Watson & Bunch 2009)
Horses	Rabies, parasitic migration, viral encephalomyelitis and infection with *Streptococcus equi* subsp. *equi*	(Metre & Mackay 2015) (Radostits *et al.* 2010 p. 771)
Sheep	Invasion of brain tissue by the intermediate stage of *Taenia multiceps* (i.e. *Coenurus cerebralis*)	(Metre & Mackay 2015)
General	Space occupying masses (e.g. tumours, abscesses)	(Metre & Mackay 2015)

De Tores et al. (2007) reviewed various studies (Burbidge & McKenzie 1989 ; Dickman 1992 ; Johnson, Burbidge, & McKenzie 1989), and concluded that data available does not suffice to conclude that disease was the only factor responsible for the historical decline of *S. brachyurus*. Nonetheless, the authors advise against trivialising the possible effects that infectious disease may have had on natural populations. Although inconclusive and speculative, it was certainly possible that disease was not the only factor responsible for the decline of *S. brachyurus* on the mainland, however, considering the resemblance of field observations to clinical signs found in known and already described disease processes, it seems reasonable to infer that quokkas on the mainland have indeed experienced infectious disease. Furthermore, some of these field observations correspond to clinical findings that could easily have passed unnoticed if no proper surveillance was in place.

Although it is likely that multiple factors may have played a synergistic role in the decline of the quokka in the 1930s, reports indicating mass death events due to disease go back as far as 1901, well before the European red fox had penetrated the historical geographical distribution of the quokka (i.e. Geraldton in 1925) (King & Smith 1985). It is not inconceivable to consider that the decline of the quokka on the mainland had already been in motion by the time the European red fox fully colonised most of the southwest of WA (King & Smith 1985). It is not possible to study these mass mortalities in any detail but it should be assumed that whatever the cause was could occur again and similarly affect the current populations.

In this context, the current absence of health and disease surveillance in the quokka is a concern in the conservation strategy of this species. Underestimating what infectious diseases can do, while stress builds up in local wildlife populations due to other pressures (e.g. climate change, clearing of natural habitat) could result in disease outbreaks that irrevocably effect extant populations. In the case of *S. brachyurus*, health and disease surveillance has been overlooked entirely. Although there is no information on diseases in free-ranging quokkas, there is still a considerable number of studies that have examined quokka diseases and agents of disease in captive animals. A review of these studies follows.

2.4 Diseases in *S. brachyurus* review

Research indicates that disease has the potential to cause significant wildlife populations declines and even species extinction (Aguirre & Tabor 2008 ; Daszak & Cunningham 1999 ; Pedersen *et al.* 2007). Preventive management and mitigation of such impacts, is not just about active surveillance, but also about knowing the species being managed (ex-situ and in-situ), knowledge that includes agents of disease and diseases that have been linked to the species. Such data if available, would be easier to studied, managed and used, if instead of being scattered across decades of research, and across scientific journals of all sorts, is presented and collated in a concise and single document. This is the case of the quokka, were diseases and agents of disease have been reported since as early as 1914, but the information is too scattered to clearly appreciate how much is known, and too scattered to be useful in any context.

This section's particular purpose then, is to provide a comprehensive collation of diseases and agents of disease that have been previously reported to occur in quokkas (with the exception of *Salmonella* spp.). Consequently, this section does not contain information on

the main infectious agents studied in this project, that constituted chapters (i.e. *Salmonella* spp., *Cryptococcus* spp., and herpesvirus), nor on other findings such as microfilaria, *Babesia* sp., *Theileria* sp., trypanosomes, *Eimeria* sp., and nematodes which were used as supplementary data in other analyses; instead, a review of these infectious agents is provided in their corresponding chapters. The data for the review that follows were obtained from either peer reviewed literature or third party databases that hold veterinary pathology records. Although a great deal of effort was put into including as many reports as possible, there may still be diseases and agents of disease that have affected quokkas either in the wild, captivity or both that were not obtained, and thus are not mentioned here. Despite this, the following information is perhaps the first and most complete review of diseases and agents of disease in the quokka.

2.4.1 *Mycobacteriosis*

Mycobacterium is a genus in the family Mycobacteriaceae. These organisms are aerobic, non-motile, non-spore forming acid-fast bacilli, and include opportunistic environmental saprophytes and obligate pathogens than can live for extended periods of time in the environment (Biet *et al.* 2005 ; Markey *et al.* 2013 pp. 161-176). Environmental mycobacteria are found in water, soil and vegetation (Quinn *et al.* 2011 pp. 250-262). Historically, the nomenclature of mycobacteria was restricted to tuberculous (those in the *Mycobacterium tuberculosis complex*) or non-tuberculous (NTM, those of the *Mycobacterium avium complex*, and other environmental and non-obligate mycobacteria), according to Runyon's grouping [4] (Markey *et al.* 2013 pp. 161-176; Rastogi, Legrand, & Sola 2001). However, today Runyon's classification and characterisation of mycobacteria is insufficient to specify the exact taxonomic status of newly-described species. For this reason, new molecular techniques such as polymerase chain reaction-restriction fragment length polymorphism, DNA probing and whole genome sequencing are now being used (Choo *et al.* 2016 ; Garcia & Gola 2016 ; Quinn *et al.* 2016 pp. 54-57; Rastogi, Legrand, & Sola 2001) not just because they allow for the determination of species, but also subspecies and subtypes of mycobacteria.

The route of transmission varies according to the species involved, however, recognised routes of transmission include: respiratory, oral and through skin lesions (Atkins & Gottlieb 2014 ; O'Reilly & Daborn 1995). Although pathogenic mycobacteria show a considerable degree of host-specificity, other hosts can still be infected and developed disease (Olsen, Barletta, & Thoen 2010 ; Quinn *et al.* 2011 pp. 250-262). Mycobacteria

[4] Ernest Runyon (1959) grouped NTM in four groups (I, II, III and IV) according to their growth rate and colony morphology on culture media, and pigmentation either produced in the absence of light or after exposure to it.

have been typically associated with tuberculosis in a variety of hosts including humans, primates, dogs, cattle, psittacines, and sheep (Markey *et al.* 2013 pp. 161-176; Songer & Post 2005). In wildlife, it has been associated with badgers, possums, wild felids, the red fox (*Vulpes vulpes*) and wild ungulates (Biet *et al.* 2005 ; Markey *et al.* 2013 pp. 161-176; Quinn *et al.* 2011). Additionally, mycobacteria have been associated with leprosy in armadillos and chimpanzees (Donham & Leininger 1977 ; Leininger, Donham, & Rubino 1978 ; Smith *et al.* 1983 ; Truman *et al.* 1986).

In Australian marsupials, mycobacteriosis has been associated with different *Mycobacterium* species, including *M. tuberculosis*, *M. bovis*, *M. paratuberculosis*, *M. ulcerans* and others (Ladds 2009). However, members of the *M. avium complex* appear to be the principal cause of mycobacterial disease in macropods (Vogelnest & Portas 2010). The first known report of mycobacterial infection in a marsupial dates from 1895 and was recorded on a captive brushtail possum (*Trichosurus vulpecula*) in India (Moore 1903). The disease is considered to occur across Australia and most commonly affects captive populations (Vogelnest & Portas 2010), with only one known case in a free-ranging long-footed potoroo (*Potorous longipes*) (Fowler & Mitchell 2002). Multiple clinical presentations of infection with *Mycobacterium* spp. have been reported, including osteomyelitis (Mann, Montali, & Bush 1982), pulmonary mycobacteriosis (Michael & Sangster 2010), granulomatous myelitis and meningitis (Schoon *et al.* 1993), and cutaneous infection (McOrist *et al.* 1985). Species reported to be affected by mycobacteria include those belonging to the Macropodidae [e.g. the tammar wallaby (*Macropus eugenii*), swamp wallaby (*Wallabia bicolor*), western grey kangaroo (*Macropus fuliginosus*), rufous hare-wallaby (*Lagorchestes hirsutus*) and the red kangaroo (*Macropus rufus*)], Potoroidae [e.g. bettongs (*Bettongia* spp.) and the Gilbert's potoroo (*Potorous gilbertii*)], Myrmecobiidae [e.g. the numbat (*Myrmecobius* spp.)] and, Peramelidae [e.g. the northern brown bandicoot (*Isoodon macrourus*)] families. It has also been reported in the koala (*Phascolarctos cinereus*) and the Tasmanian devil (*Sarcophilus harrisii*) (Ladds 2009).

An interesting case is that of *M. bovis* infection in brushtail possums in New Zealand (NZ). *Mycobacterium bovis* occurs naturally in NZ and *T. vulpecula* (an introduced species) acts as a reservoir and vector of bovine tuberculosis to cattle (Buddle & Young 2000 ; Caley *et al.* 1999). Generally, the infection curses with lesions in lymphnodes and lungs, but masses can develop in any visceral organs (Ladds 2009). Infection studies in *T. vulpecula*, have shown that tuberculosis quickly becomes systemic and progresses into fatal disease (25-100 days post inoculation) (Jackson *et al.* 1995). By contrast, infection with *M. bovis* has not been detected in Australian possums, and any granulomatous lesions in the

species are meant to be reported to Wildlife Health Australia (https://www.wildlifehealthaustralia.com.au/) (Johnson & Hemsley 2010 pp. 395-437).

As a generalisation, metatherians (such as marsupials) are known to be more susceptible to mycobacterial infections than eutherian animals, primarily for their inability to mount a granulomatous response strong enough to control the proliferation of *Mycobacterium* spp., which according to Buddle and Young (2000), indicates a deficient interaction between lymphocytes and macrophages. According to Ladds (2009), although the condition is normally chronic and clinical signs depend on the organs affected, mycobacteriosis in wildlife is typically progressive and is often fatal.

In the quokka, the only known reported cases date from 1964 and 1982. Kakulas (1964) describes two captive quokkas, a male from Bald Island and a female from Rottnest Island, which were part of a study on nutritional myopathy and were reported to have encapsulated masses. In both cases, apparently healthy animals were found dead without apparent signs of disease. At post-mortem, gross examination showed single encapsulated masses located within the superior mediastinum (Bald Island animal), and within the mesentery in close association with the stomach and the small intestine (Rottnest Island animal). Histologically, both masses were characterised by a poorly vascularised fibrous wall, with an inner layer characterised by inflammatory tissue and mononuclear cell infiltrates, and a central zone of necrotic tissue. In both masses, the stained (Ziehl-Neelsen) necrotic tissue revealed acid-fast bacilli. Microbiological studies were performed and organisms were considered to be NTM, however, assigning these organisms to one of the Runyon's groups was not achieved and organisms were believed to be either group I, II or III. Interestingly, Peet et al. (1982) reported almost 20 years later, a case of disseminated mycobacteriosis by *M. intracellulare* serotype 42 in a tammar wallaby, that was captive at the same facility as the animals reported by Kakulas in 1964. This case followed that of a quokka that had previously been studied, in which mycobacteriosis was suspected. Acid-fast stained bacilli were observed in histopathological lesions from a mediastinal lymph node. The organisms were not fully identified due to the absence of fresh material for culturing.

2.4.2 *Encephalomyocarditis virus*

Encephalomyocarditis virus (EMCV) is one of the two species in the *Cardiovirus* genus and belongs to the *Picornaviridae*. Picornaviruses are single-stranded positive-sense Ribonucleic Acid (RNA) viruses with no envelope, of an approximate diameter of 30 nm

(MacLachlan & Dubovi 2011 pp. 3-19). Experimentally, transmission of EMCV can be demonstrated by any route of exposure: oral, aerosol, subcutaneous, intramuscular, intranasal, intratracheal, intracranial (Zimmerman 1994) and intravenous (Thompson, Bengis, & Brown 2001). Viral transmission via wound contamination that was proposed as a possible route to humans, was later demonstrated to be an efficient route in swine (Zimmerman 1994). The usual route of transmission is faecal-oral although it may also occur by aerosols and fomites (Quinn *et al.* 2011). Though not contagious in rodents (Thompson, Bengis, & Brown 2001), contact transmission among caged animals has been reported and was attributed to fighting and cannibalism (Tesh & Wallace 1978). In pigs, two mechanisms of natural transmission have been postulated: 1. after ingestion of contaminated faeces or the carcasses of infected rodents (Billinis 2009 ; Maurice *et al.* 2005); or 2. horizontal or vertical transmission (Maurice *et al.* 2005).

Encephalomyocarditis virus has been reported in both captive (Citino *et al.* 1988 ; Wells *et al.* 1989) and wild animal species (Amaddeo, Cardeti, & Autorino 1995 ; Gainer & Bigler 1967 ; Grobler *et al.* 1995). However, the virus is more prevalent in rodents, who present discrete infections (Thompson, Bengis, & Brown 2001). Consequently, the virus is regarded as a rodent virus (MacLachlan & Dubovi 2011 p. 438; Maurice *et al.* 2005). Some authors suggest that the high prevalence of EMCV in rodents is just an indicator of the circulation of the virus among animal species in a given environment (Tesh & Wallace 1978 ; Zimmerman 1994). Encephalomyocarditis virus may cause isolated and sporadic outbreaks of myocarditis and sudden death in a range of species, but particularly in wild animals in captivity (Thompson, Bengis, & Brown 2001). Among domestic animals, pigs are considered to be the most susceptible species (Maurice *et al.* 2005) but other domestic species such as horses, cats, cattle and dogs are also susceptible (Thompson, Bengis, & Brown 2001 pp. 124-130). This virus appears to be present worldwide (Grobler *et al.* 1995). This virus has also been isolated from humans; however, the disease is very infrequent (Oberste *et al.* 2009).

Members in the family *Picornaviridae* can be highly pathogenic and infectious, and are responsible for a wide range of conditions, ranging from common cold-like clinical disease to life threatening encephalomyelitis and myocarditis (MacLachlan & Dubovi 2011 p. 425; Semler & Ertel 2010 p. 565). Clinical disease expression is dependent on virulence of EMCV strains (i.e. lethal or non-lethal strains), as well as host susceptibility (Zimmerman 1994). Most EMCV-infected animals are presented with sudden death with no prior signs of illness (Reddacliff *et al.* 1997 ; Thompson, Bengis, & Brown 2001). In less sudden cases, clinical signs are generally related to acute congestive heart failure, pulmonary congestion

and oedema, hydropericardium and ascites. They include listlessness, moderate to severe dyspnoea, anorexia (Thompson, Bengis, & Brown 2001) and laboured respiration (Carocci & Bakkali-Kassimi 2012).

In Australia, EMCV was isolated from an apparently healthy water-rat (*Hydromys chrysogaster*) in north Queensland (QLD) (Pope 1959) and came to be the first report of natural infection with a virus of the encephalomyocarditis group in Australia. The virus has also been reported in pigs (Links *et al.* 1986 ; Seaman, Boulton, & Carrigan 1986), cattle (Diallo, Carter, & Storie 2013), humans (Kirkland *et al.* 1989), free-ranging common rats (Reddacliff *et al.* 1997), and a variety of exotic and native wildlife in captivity (Moloney 2001 ; Reddacliff *et al.* 1997). Between 1987 and 1995, samples were collected from various animals at post-mortem at Taronga Zoo, including apparently healthy rats (*Rattus rattus* and *R. norvegicus*) and mice (*Mus musculus*) that were not part of the collection. Encephalomyocarditis virus was isolated from two Goodfellow's tree kangaroo (*Dendrolagus goodfellowi*) and one rat, as well as from other species exotic to Australia (Reddacliff *et al.* 1997). The virus has also been isolated from three captive common wombats (Vombatus ursinus) that the died suddenly without any observable signs of illness (McLelland 2000 ; Moloney 2001). The Australian Registry of Wildlife Health (ARWH) recorded two confirmed cases of EMCV in tammar wallabies (*M. eugenii*), and possible cases in a range of other marsupials including eastern (*Macropus giganteus*) and western grey kangaroos (*Macropus fuliginosus*) and ringtail (*Pseudocheirus peregrinus*) and brushtail possums (*Trichosurus vulpecula*). As per the commonly reported profile of disease manifestation, the two tammar wallabies were apparently healthy and did not exhibit signs of disease.

According to the literature, EMCV has only been diagnosed one time in *S. brachyurus*. A retrospective study by McLelland (2000) investigated the presence of EMCV antigen in formalin-preserved tissue samples using an immunoperoxidase staining technique. Archived samples that were collected from captive individuals (various species) between 1980 and 1998, from animals in which acute myocarditis was diagnosed, were retrieved from the Australian Registry of Wildlife Pathology at Taronga Zoo, NSW Australia. The only animal that was found to be infected with the virus was from Perth Zoo and was one of a group of four quokkas, all individuals with a history of elevated creatine kinase and alanine aminotransferase. In line with the most common clinical presentation of EMCV (Reddacliff *et al.* 1997 ; Thompson, Bengis, & Brown 2001), the animal died suddenly with no clinical signs of disease. At post-mortem, pericardial fluid and a very pale myocardium were observed, as well as congestion in abdominal viscera, and lungs were oedematous.

Histopathology studies revealed extensive myocardial necrosis and degeneration, as well as extensive skeletal myopathy with degeneration of fibres and mineralisation. Both the myocardium and skeletal tissues presented with a mononuclear inflammatory infiltrate. Positive intracellular immunoperoxidase staining was identified in myocardial cells.

2.4.3 *Poxvirus*

The family *Poxviridae* has two subfamilies, however only the subfamily *Chordopoxvirinae* is of relevance to this review, as it comprises the poxviruses (PVs) of vertebrates (MacLachlan & Dubovi 2011 p. 152; Robinson & Kerr 2001). These viruses are very resistant and can persist in the environment for years (Rheinbaben *et al.* 2007). There are a number of unclassified PVs related to wildlife, with the significant one being the macropodid poxvirus (Robinson & Kerr 2001) that, like most PVs, resemble orthopoxviruses – being large from 200 to 400 nm in length, rectangular or brick-shaped and covered with irregular tubular elements (Ladds 2009 ; Robinson & Kerr 2001). (Ladds 2009 ; Robinson & Kerr 2001). There are many routes for poxviral transmission and they include respiratory (e.g. V*ariola virus*), percutaneous inoculation from vectors (e.g. insects in the *Myxoma virus*), oral mucosa or skin penetration via abrasions (e.g. parapoxviruses) (MacLachlan & Dubovi 2011 ; Robinson & Kerr 2001).

Poxviruses have a worldwide distribution, including Australia, and have a broad host range (Robinson & Kerr 2001). Many PVs and their wildlife hosts appear well adapted to each other and they are often only detected when they spill-over from their natural hosts to susceptible definite hosts such as people, or in domestic animals were disease becomes apparent when animals are intensively managed (e.g. *Parapoxvirus* of red deer in New Zealand) (Robinson & Kerr 2001). Other conditions where viruses may be picked up include wild animals in captivity (e.g. *Parapoxvirus* infection in captive seals in Germany) (Müller *et al.* 2003) or in laboratory colonies (e.g. yatapoxviruses in primates in USA) (Rheinbaben *et al.* 2007). As a result of this, little is known about many PVs of wild animals under natural conditions, with many discoveries being incidental or opportunistic, or inferred from laboratory studies or field surveys. Poxviruses display variation in pathogenicity in different hosts (as a result of differences in host immunity), as the same virus can cause a localised infection in one host, but lead to generalise disease in another. This is still poorly understood (McFadden 2005 ; Robinson & Kerr 2001) but a good example of this is the infection with *Myxoma virus* that results in a benign fibroma in the rabbit (*Sylvilagus brasiliensis*), but causes the lethal disease myxomatosis in the European rabbit (*Oryctolagus cuniculus*) (Kerr & Best 1998). Zoonotic infections with most of these

viruses are occupational diseases occurring in veterinarians and animal handlers (e.g. Cowpox virus and Vaccinia virus), or from eating or handling wild animals (e.g. Monkeypox virus from eating squirrels and monkeys) (MacLachlan & Dubovi 2011 p. 157).

In Australia, PVs have been mostly detected in macropods (Ladds 2009). Generally, rates of infection appear to be low and the disease is relatively mild (Ladds 2009 ; Vogelnest & Portas 2010 p. 166). On a whole, Australian PVs have not been properly characterised, apart from an *Orthopoxvirus* outbreak in the common ringtail possum (*Pseudocheirus peregrinus*) (Vogelnest, Stewart, & Sangster 2012). And while there is almost a lack of information on species specificity or transmission, the virus is thought to occur wherever suitable hosts are present (Vogelnest & Portas 2010 p. 183). It is generally believed that macropod PVs are species-specific, as observed in a captive colony of eastern grey kangaroos (*Macropus fuliginosus*) where only this species was infected and newly introduced eastern grey kangaroos developed the disease, but other species did not (Speare 1988b). In Australia, there is no target surveillance or management program for PVs but cases of PVs in native wildlife are logged in the national database by the general wildlife health surveillance system. Little is known about the PVs reported in the National Wildlife Health Surveillance Database, and it is likely that infection in native wildlife is more common than the reported figures.

From the literature, Australian native mammal species reported with poxvirus (PV) infection include macropods such as the western grey kangaroo (*Macropus fuliginosus*), tammar wallaby (*Macropus eugenii*) and the quokka (*Setonix brachyurus*) (Arundel, Beveridge, & Presidente 1979 ; Bagnall & Wilson 1974 ; Ladds 2009 ; Ladds 2012 ; McKenzie, Fay, & Prior 1979 ; Papadimitriou & Ashman 1972 ; Reece & Hartley 1994 ; Rothwell *et al.* 1984). Other marsupials include the common brushtail possum (*Trichosurus vulpecula*) (Samuel 1989), short-beaked echidna (*Tachyglossus aculeatus*) (Whittington 1993); with anecdotal reports in a Tasmanian pademelon (*Thylogale billardierii*) filed in the database of the electronic Wildlife Health Information System (eWHIS) . Transmission of the virus in Australia is likely through arthropod vectors or direct transfer via close contact between individuals in a group (Vogelnest & Portas 2010). Generally, control of the disease in wild populations is difficult and one focus could be on reducing arthropod vectors (Bray 2011).

In macropods, infections can occur in all ages, but usually in juvenile animals or subadults (Ladds 2009). Clinical signs include skin lesions (solitary or multiple coalescing) that vary in size from a few millimetres up to 5 cm. More commonly, these proliferations are

characterised by irregular-shaped wart-type masses with a hyperkeratotic surface and a central umbilicated crater that becomes more hairless and darker as the mass enlarges (Vogelnest & Portas 2010). This epidermal thickening is due to acanthosis and hyperkeratosis. With this form, lesions regress spontaneously over a few months with no associated pruritus (Vogelnest & Portas 2010). Lesions may be secondarily infected by fungi and bacteria that lead to infiltration of leukocytes. Another form includes firm umbilicated papules containing exudates of keratinised debris and purulent material (Reece & Hartley 1994 ; Speare 1988b). Although most lesions have been described on the tails of different species, they can be found anywhere on the body (Vogelnest & Portas 2010). Apart from the skin lesions that are usually benign, infected macropods appear otherwise healthy (McKenzie, Fay, & Prior 1979). Surgical excision may be necessary if lesions impede the animal's health (e.g. near eyes or mouth) (Vogelnest & Portas 2010). Macropod marsupials with cutaneous lesions containing PVs have been recorded to occur in eastern and western Australia (Rothwell *et al.* 1984).

Poxviral infection in the quokka was first reported by Papadimitriou and Ashman (1972). The population studied (i.e. Rottnest Island), presented animals with raised epidermal lesions associated with poxvirus; these lesions were either single or multiple and commonly found on the dorsal aspect of the tail, and varied in size from a few millimetres to 4-5 cm (Papadimitriou & Ashman 1972). According to the authors, these lesions were quite prevalent. Their histological studies showed that papilloma-like lesions in *S. brachyurus* were non-malignant and presented with hyperkeratosis and acanthosis, as well as vacuolated cells in the stratum granulosum containing eosinophilic cytoplasmic inclusions displacing their nucleus; inclusions that corresponded to viral particles resembling immature and mature PVs by electron microscopy (Papadimitriou & Ashman 1972). In 1983, Stanley and Mackenzie were able to confirm that the electron microscopy observations of Papadimitriou and Ashman (1972) in fact corresponded to a PV. Furthermore, complement-fixation studies showed a mild cross-reaction between the PV present in skin lesions of quokkas, and *Fowlpox virus* (FWPV), which lead the authors to suggest that this virus (in quokkas) may be a member of the *Avipoxvirus* genus (Stanley & Mackenzie 1983). However, this is very unlikely. This cross-reactivity between FWPV and the PV detected in *S. brachyurus* may be the result of genetic similarities between avian PVs and mammalian PVs, but still sufficiently phylogenetically different to be different viruses. An example of this, is the more than 49% homology between thymidine kinases amino acid sequences of FWPV and four mammalian PVs (Gibbs 1987). Moreover, there is strong evidence supporting that members of the *Avipoxvirus* are host specific and only infect avian species (Gubser *et al.* 2004 ; Gyuranecz *et al.* 2013 ; Jarmin *et al.* 2006 ;

Skinner 2010). Although there is limited data on the species specificity of PVs in Australian fauna, the observations of Speare (1988b) may suggest that this as yet uncharacterised PV may be specific to the quokka.

In 2010, the report "Threatened fauna species management plans for ALCOA's bauxite mining operations in the Jarrah forest" (Stokes & Norman 2010), expressed concern of a possible cross infection of *S. brachyurus* with *Canarypox virus* that was used as a vector platform for an equine influenza virus vaccine that was extensively used during the equine influenza virus outbreak in Australia during 2007. This was subsequently included in recent publications dealing with the conservation status and management of the quokka (de Tores *et al.* 2007 ; Department of Environment and Conservation 2013). However, there is no basis for this concern. According to "Equine Influenza, The August 2007 Outbreak in Australia", a report of the Equine Influenza Inquiry launched by the Australian Federal Government (Callinan 2008), vaccination was only approved in nominated buffer zones across New South Wales (NSW), Victoria, and QLD not WA. Furthermore, according to Cowled et al. (2009), the outbreak was effectively restricted to two eastern states QLD and NSW. Additionally, *Canarypox virus* is a member of the genus *Avipoxvirus*, which infects only non-mammalian hosts (Bray 2011 p. 399; Moss 2001 ; Skinner 2010). Moreover, recombinants of *Canarypox virus* used in vaccines carrying antigens from mammalian pathogens, do not replicate in mammalian cells which precludes dissemination of the agent (Animal Health Australia 2011 ; Poulet *et al.* 2007).

Even though reports of poxviral disease in Australian fauna are scarce in the literature, PVs are likely to be widespread in marsupials. Based on the available information, PVs do not appear to pose a threat to free-ranging *S. brachyurus* on Rottnest Island, which also appears to be the case of cross-infection with the recombinant *Canarypox virus* vector previously mentioned. On the mainland, extensive trapping on the northern and southern sub-populations appear to indicate that lesions resembling those described by Papadimitriou and Ashman (1972) are not present (S. Dundas, *pers. comm.* 2013; K. Bain, *pers. comm.* 2013). However, increasing global temperatures may potentially lead to increased vector activity and numbers. In turn, this may result in a higher incidence and prevalence of poxviral infections.

2.4.4 ***Arthropod-borne viruses***

2.4.4.1 Arboviruses

Arboviruses (AVs) or arthropod-borne viruses refer to a group of viruses that are transmitted biologically between vertebrate hosts via haematophagous arthropod vectors, such as mosquitoes, ticks, midges and other biting flies (Hubálek, Rudolf, & Nowotny 2014 ; Muller 1995 ; Russell & Dwyer 2000 ; Weaver & Reisen 2010). Prior to transmission, AVs replicate in arthropod vectors and then transmit through the saliva of the vector upon biting a host species (Russell & Dwyer 2000 ; Weaver & Reisen 2010). The arboviral group is comprised of a wide range of RNA viruses: (*Togaviridae*: alphaviruses), (*Flaviviridae*: flaviviruses), (*Bunyaviridae*: bunyaviruses, nairoviruses, phleboviruses), (*Reoviridae*: orbiviruses), (*Rhabdoviridae*: vesiculoviruses) and (*Orthomixoviridae*: thogotoviruses), while *African swine fever virus* is the only DNA arbovirus (Weaver & Reisen 2010). All AVs circulate among wildlife populations. They cause disease in humans and domestic animals, which are usually dead-end or incidental hosts, after 'spill over transmission' from wild animal populations; as a result, they have increased in importance as both veterinary and human pathogens (Markey *et al.* 2013 ; Weaver & Reisen 2010).

In Australia, AVs have been recorded to be widespread in both humans and animals (Coffey *et al.* 2014 ; Doherty 1972 ; Ladds 2009 ; Stanley & Mackenzie 1983). Out of the more than 70 AVs reported in Australia, only a small number are pathogens of humans and even fewer are of major public or veterinary health concern (Aaskov & Doherty 1994 ; Mackenzie *et al.* 1998 ; Russell & Dwyer 2000 ; Stanley & Mackenzie 1983), however, their medical significance appears to be increasing (Coffey *et al.* 2014). Significant examples include *Ross River virus*, *Barmah Forest virus*, *Dengue virus*, *Murray Valley encephalitis virus*, *Japanese encephalitis virus* and *West Nile virus* (Ladds 2009 ; Mackenzie *et al.* 1994 ; Russell & Dwyer 2000). All these viruses are typically zoonotic and associated with rural areas, where seroprevalence is generally high (Russell & Dwyer 2000). This section will only focus on *Ross River* (RRV), *Barmah Forest* (BFV) and Trubanaman (TRUV) viruses, as they were detected in WA in the quokka (Johansen *et al.* 2005 ; Lindsay 1995).

Both RRV and BFV, members of the *Togaviridae*, are icosahedral, with a diameter of 65-70 nm, and comprise an enveloped liner, single positive-sense RNA strand of 11-12 kb, as well as sub-genomic mRNA. In contrast, TRUV is characterised by a single negative-sense RNA with three segments (Walter & Barr 2011). While *Ross River virus* and BFV have relatively restricted geographic ranges, and are considered to be endemic to Australia

(Jacups, Whelan, & Currie 2008 ; Mackenzie *et al.* 2001), TRUV a member of the *Bunyaviridae*, has been isolated in Australia and Papua New Guinea (Plyusnin *et al.* 2012). Mosquitoes of the Aedes, Culex, and Anopheles genera are the most important vectors for these three viruses in WA (Jacups, Whelan, & Currie 2008 ; Stanley & Choo 1964). In coastal regions, principal vector species of both RRV and BFV include *Aedes vigilax* and *A. camptorhynchus*, while in inland areas, *Culex annulirostris* is the major mosquito vector (Naish *et al.* 2006 ; Russell & Dwyer 2000). Only one vector has been found to carry and transmit TRUV, *Anopheles annulipes* (Doherty 1972 ; Johansen *et al.* 2005). Although other species may be involved in the amplification of RRV, BFV and TRUV (Johansen *et al.* 2005 ; Russell 2002), marsupials particularly kangaroos and wallabies, are believed to be the major vertebrate reservoir hosts for these three viruses (Carver *et al.* 2009 ; Jacups, Whelan, & Currie 2008 ; Jeffery *et al.* 2002 ; Johansen *et al.* 2005 ; Kay *et al.* 1986 ; Mackenzie *et al.* 1998).

In the southwest of WA, RRV, BFV and TRUV viruses have often been isolated from mosquitoes as part of a monitoring program by the Arbovirus Surveillance and Research Laboratory, and the seroprevalence of the viruses has been recorded to be high in marsupials (Johansen *et al.* 2005 ; Lindsay 1995). These three viruses were detected in *S. brachyurus* through neutralising antibody studies using sera (Johansen *et al.* 2005 ; Lindsay 1995). A study by Lindsay (1995) recorded four quokkas having antibodies to RRV and suggested that the species is occasionally infected and bitten by mosquito vectors. While studying more than 3,000 sera collected from 20 animal species, Johansen et al. (2005) detected neutralising antibodies to BFV in quokkas with a prevalence of 3.2% (2/62) in the southwest region of WA. This prevalence was the highest overall, followed by horses (1.2%) and humans (0.9%). When examining the Peel and south coastal localities, where quokkas were sampled, the highest prevalence of neutralising antibodies to BFV was that of quokkas (11%, 2/19) followed by horses (1.4%) (Johansen *et al.* 2005). This made the authors suggest that quokkas may have been involved in viral transmission of BFV in the southwest of WA given that most human cases of disease caused by BFV infection between 1992 and 1994, occurred in these two areas. The same study detected neutralising antibodies to TRUV in one quokka of 62 studied (prevalence 1.6%) (Johansen *et al.* 2005). A study by Stanley (Stanley 1975) found that 65% of the 87 Rottnest Island quokka sera tested had antibodies to flaviviruses (a group that includes the *Murray Valley* and *Japanese encephalitis* viruses), however, the virus was not identified.

With the exception of RRV experimental infections in brushtail possums (*T. vulpecula*) where infected animals exhibited non-specific signs of disease such as lethargy,

inappetence and unsteady gait, four days post infection (Boyd *et al.* 2001), little is known about the clinical signs of disease that RRV, BFV and TRUV could cause in native species in the wild, if any. Though viraemia has been observed in many marsupials infected with AVs, no definite disease – overt illness or demonstrable lesions - have been associated with the presence of RRV, BFV, and TRUV infections (Ladds 2012 ; Munday 1976).

2.4.4.2 Reoviruses

The family *Reoviridae* comprises 15 genera, of which only members of *Coltivirus*, *Orthoreovirus* (ORV), *Orbivirus* (ORBV), *Rotavirus* (RTV), *Seadornavirus*, and *Aquareovirus* infect man and animals (MacLachlan & Dubovi 2011). Though today, reoviruses (RVs) are responsible for disease in many species, the acronym 'reo' was originally derived from 'respiratory'; 'enteric'; 'orphan' viruses. The reason being that it was isolated from respiratory and enteric samples, but was not associated with clinical disease (i.e. orphan virus) (Kapikian & Shope 1996). Members of *Reoviridae*, are ubiquitous, have a genome of segmented (9-12) linear double-stranded RNA (ds-RNA), are structurally icosahedral, have a double capsid that lacks an envelope, and are of approximately 60-85 nm in diameter (Coombs 2010 ; MacLachlan & Dubovi 2011). Orthoreoviruses (ORVs) and rotaviruses are primarily transmitted via contact with contaminated faeces, either directly or indirectly (Kapikian & Shope 1996). Rotaviruses cause significant gastroenteric disease in humans and intensively reared farm animals (Kapikian & Shope 1996 ; MacLachlan & Dubovi 2011), while ORVs are known to cause significant disease in squamates (Latney & Wellehan 2013). Orbiviruses (ORBVs) are transmitted most commonly via arthropod vectors of the *Culicoides* genus, though ticks, black flies, sandflies and mosquitoes can also function as vectors for ORBVs (MacLachlan & Dubovi 2011). These viruses cause a number of significant animal diseases including African horse sickness and Bluetongue disease in ruminants (Kapikian & Shope 1996 ; Markey *et al.* 2013).

In marsupials, particularly macropods, three species of ORBVs have been linked with serious disease. Viral chorioretinitis caused by *Wallal* and *Warrego* viruses both ORBVs, appeared in NSW between April and July 1994, and by April 1996 the disease had spread to WA affecting thousands of animals (Durham *et al.* 1996 ; Hooper *et al.* 1999 ; Reddacliff 2012). These RVs however, were detected by PCR in an archived sample from a blind kangaroo in 1975 (Vogelnest & Portas 2010). Species most commonly affected included western and eastern grey kangaroos, euros and red kangaroos (Hooper *et al.* 1999), with the most common clinical sign being blindness accompanied by uveitis and occasional conjunctivitis (Reddacliff 2012 ; Vogelnest & Portas 2010). It is believed that the eye

lesions resulting in loss of sight, are not directly inflicted by these viruses; instead, lesions are most likely immune mediated (Reddacliff 2012). *Eubenangee virus* an ORBV, was isolated from tissue samples including cerebrospinal fluid and cerebral cortex of Tammar wallabies that died without obvious clinical signs of disease over a short period of time, often described as *sudden*, at various institutions holding animals in captivity in eastern Australia. Due to the acute presentation of this condition, the disease was termed "Tammar sudden death syndrome" (Rose *et al.* 2000).

Early reoviral studies in the 1960s in WA detected antibodies to Reovirus serotypes 1, 2 and 3 in the sera of quokkas (Stanley & Leak 1963 ; Stanley *et al.* 1964). Today, Reovirus serotypes 1, 2 and 3 are known as the *Mammalian orthoreovirus* species from the ORV genus of *Reoviridae*.

Mammalian orthoreovirus (MORV) and its multiple serotypes, are ubiquitous and are known to infect both humans and animals and while infections occur frequently, these are generally not clinically significant (Clarke & Tyler 2010 ; Wellehan *et al.* 2009). Interestingly, rodents infected with MORV-3 develop disease with a clinical presentation similar to that in humans with extrahepatic biliary atresia, also accompanied by damage to the CNS (Clarke & Tyler 2010). Although rare, there have also been isolated reports of meningitis, encephalitis, pneumonia and keratoconjunctivitis in humans attributed to infection with serotype 3 (Clarke & Tyler 2010). However, MORV and its multiple serotypes has been detected in patients with conditions affecting almost all organ systems, and it has been recover in faeces, blood, urine, pharyngeal and nasal secretions, cerebrospinal fluid and organs (Kapikian & Shope 1996). Overall, this ubiquity makes it more difficult to associate MORV infection with clinical disease syndromes, hence, the role of these viruses in disease remains unclear (Clarke & Tyler 2010 ; Kapikian & Shope 1996 ; Stanley *et al.* 1964). Serologically identical strains to the human reovirus serotypes have been isolated from a wide variety of animals such as dogs, cats, rats, sheep, cattle, horses and swine (Kapikian & Shope 1996 ; Stanley & Mackenzie 1983).

A study done by Stanley and Leak (1963) with 72 free-ranging *S. brachyurus* from Rottnest Island and the mainland of WA showed that MORV-seropositive individuals were more prevalent in areas where there is continuous contact with people, compared to areas with little human contact (Stanley & Leak 1963 ; Stanley & Mackenzie 1983). The study aimed to determine seroprevalence of antibodies to MORV in some Australian mammals and whether humans constitute the main reservoir and animals that come into contact with humans or their immediate environment may become infected and develop antibodies.

Greater prevalences for all serotypes of MORV were obtained in quokkas captured in settled areas on Rottnest Island (MORV-1: 67%, 18/27; MORV-2: 89%, 24/27; MORV-3: 93% 25/27) as well as on individuals translocated to the mainland (MORV-1: 70%, 7/10; MORV-2: 100%, 10/10; MORV-3: 100%, 10/10), compared to animals captured in areas with less human contact (MORV-1: 17%, 6/35; MORV-2: 34%, 12/35; MORV-3: 28% 10/35). Similarly, there was a greater prevalence of seropositive animals to all three MORV types in settled areas (67%, 18/27) on Rottnest Island than in areas with less human contact (11%, 4/35) (Stanley & Leak 1963). Although no statistical analyses were carried out, the results suggested a positive correlation between human contact and a greater prevalence of MORV-seropositive animals (Stanley & Leak 1963).

Experimental infection (various routes of infection) in captive and newly captured quokkas was carried out in an effort to establish an epizootic, however, this was unsuccessful, and no clinical signs of disease were observed (Potkay 1977 ; Stanley & Leak 1963). Only one death related to myocarditis was recorded and attributed to this experimental reoviral infection (Stanley & Leak 1963). A later study by Stanley et al. (1964) found similar serologic evidence of reoviral exposure in quokkas on Bald Island, and in one case, MORV-3 itself was isolated from one of six faecal specimens obtained. Of the 44 animals (sera) tested by haemagglutination inhibition (HI), three (7%) presented antibodies to one serotype of *Mammalian orthoreovirus* (serotype not specified), while 24 (54.5%) showed positive HI to all three serotypes. This widespread occurrence of viral antibodies to all three *Mammalian orthoreovirus* serotypes across ecologically different and geographically distant subpopulations such as Rottnest Island and Bald Island, may simply be a reflection of the lack of host specificity of this virus, therefore permitting continuous inter-species infections (Stanley *et al.* 1964), in addition to its capacity to survive in the environment at temperatures below 37˚C and its efficient transmissibility involving secretions, respiratory aerosols and the faecal-oral route (Clarke & Tyler 2010).

2.4.5 ***Protozoal diseases***

2.4.5.1 Toxoplasmosis

Toxoplasmosis is a common zoonotic infection caused by the ubiquitous intracellular protozoan parasite *Toxoplasma gondii* in all warmed-blooded species, including macropods (Dubey & Odening 2001 ; Ladds 2009). The life cycle of *T. gondii* comprises two phases, a sexual phase that takes place in the enteroepithelial cells of any member of the family Felidae (definite host), where ingestion of tissues containing any of the three infectious forms of *T. gondii* (i.e. tachyzoites, bradyzoites and sporozoites) (Dubey 2004), results in the production and faecal shedding of environmentally resistant oocysts

(Buxton & Maley 2013 ; Dubey 2004). These oocysts will then sporulate in the environment and be ingested by susceptible animals (intermediate hosts), in which the asexual phase takes place. Sporulated oocysts then release sporozoites that will colonize the intestinal epithelium transforming then into tachyzoites (rapidly multiplying). This infectious form will then multiply asexually by endodyogeny within the cell, which will then rupture releasing organisms systemically while inducing a strong immune response (Dubey 2004 ; Dubey & Odening 2001). In response to immunity, tachyzoites differentiate into bradyzoites that will trigger the formation of thick-walled tissue cysts (more in muscular and nervous tissues) which are resistant to the immune response, hence establishing a persistent infection (Dubey 2004 ; Dubey & Odening 2001 ; Khan, Dubey, *et al.* 2011).

In marsupials, the prevalence of *T. gondii* displays interspecific variation (Canfield, Hartley, & Dubey 1990), and appears to be affected by a range of factors, including modes of feeding, climatic conditions, sex, living conditions (captive/free-ranging) and the presence of cats (Attwood, Woolley, & Rickard 1975 ; De Camps, Dubey, & Saville 2008 ; Dubey & Odening 2001 ; Eymann *et al.* 2006 ; Miller *et al.* 1992 ; Parameswaran *et al.* 2009 ; Vogelnest & Portas 2010). Infection in macropods occurs via the oral route, either by consumption of food or water that was contaminated with *T. gondii* (Dubey & Odening 2001 ; Portas 2010). However, a recent study by Parameswaran et al (2009), obtained evidence indicating that vertical transmission of *T. gondii* from the dam to the pouch young can occur in Australian macropods that are chronically infected.

Australian marsupials are particularly susceptible to *T. gondii*, where toxoplasmosis can be fatal and morbidity and mortality rates tend to be high, particularly in captive animals (Adkesson *et al.* 2007 ; Bermúdez *et al.* 2009 ; Canfield, Hartley, & Dubey 1990 ; Eymann *et al.* 2006 ; Johnson *et al.* 1989 ; Miller *et al.* 1992). Among captive macropods, there appears to be a difference in susceptibility to the agent, with wallabies often dying of toxoplasmosis, while kangaroos appear to survive the infection (Dubey & Odening 2001), however, this has been suggested to be the reflection of a greater number of wallaby species in captive collections than kangaroos (Portas 2010). Infection can manifest in a variety of ways including being unapparent or resulting in sudden death (Canfield, Hartley, & Dubey 1990). Generally, individuals are more likely to succumb to disease or present with overt disease if they are immunosuppressed (e.g. nutritional or weather stressors), by causing latent infection to become clinically obvious and subsequently fatal (Canfield, Hartley, & Dubey 1990 ; Portas 2010 ; Vogelnest & Portas 2010). Alternatively, death could be caused by exposure of previously naïve animals (e.g. through faecal

contamination of food) to the pathogen, in which rapidly multiplying tachyzoites overwhelm the immune response (Dubey & Odening 2001 ; Johnson *et al.* 1989 ; Vogelnest & Portas 2010). In some cases, where clinical signs were present, gross findings at post-mortem examination were minimal (Canfield, Hartley, & Dubey 1990 ; Dobos-Kovacs *et al.* 1974 ; Ladds 2009). Some of these clinical signs include enteric disease (e.g. weight loss, anorexia and diarrhoea), inappetence, lethargy, unnatural activity, listlessness, depression, respiratory distress (e.g. dyspnoea, pneumonia), neurological conditions (e.g. ataxia, dysphagia and incoordination) and eye pathologies (e.g. retinal damage, papilledema and cataracts) (Canfield, Hartley, & Dubey 1990 ; Dubey & Crutchley 2008 ; Ladds 2009 ; Miller *et al.* 1992 ; Patton *et al.* 1986 ; Portas 2010 ; Vogelnest & Portas 2010).

Toxoplasmosis has also been associated with reproductive failure (e.g. abortion) (Radostits *et al.* 2010 p. 1518), but in macropods, Mayberry et al. (2014) suggested that *T. gondii* may not have any influence on the reproductive performance of female western grey kangaroos, based on the absence of statistical significance between the presence of antibodies against *T. gondii* and the absence of pouch young.

Even though toxoplasmosis is treatable, a definite diagnosis is generally not made until post-mortem examination and histopathology studies are performed (Adkesson *et al.* 2007 ; Dobos-Kovacs *et al.* 1974 ; Patton *et al.* 1986). Current ante-mortem tests for the pathogen include serological tests [e.g. enzyme-linked immunosorbent assay (ELISA), direct agglutination test or DAT, and modified agglutination test or MAT], culture and bioassays, as well as histopathology (i.e. biopsy) (Hill *et al.* 2006 ; Parameswaran *et al.* 2009 ; Vogelnest & Portas 2010). Post-mortem lesions can include necrosis (e.g. ocular, hepatic, nervous or pulmonary), splenomegaly, lymphadenomegaly, pancreatic swelling, gastrointestinal reddening and ulceration, as well as pulmonary oedema, congestion and consolidation and myocardial haemorrhages (Canfield, Hartley, & Dubey 1990 ; Ladds 2009 ; Reddacliff *et al.* 1993 ; Vogelnest & Portas 2010).

The presence of *T. gondii* antibodies and free or encysted forms of the pathogen in tissues have been reported in a range of wild marsupials including macropods such as kangaroos, wallaroos and wallabies (Attwood, Woolley, & Rickard 1975 ; Bermúdez *et al.* 2009 ; De Camps, Dubey, & Saville 2008 ; Gibb *et al.* 1966 ; Johnson, Roberts, & Munday 1988 ; Johnson *et al.* 1989 ; Mayberry *et al.* 2014), bandicoots (Bettiol *et al.* 2000 ; Miller *et al.* 2000 ; Obendorf & Munday 1990), dasyurids (Attwood, Woolley, & Rickard 1975), wombats (Hartley & English 2005) and possums (Eymann *et al.* 2006 ; Hartley 1993). In

WA, *T. gondii* has been reported in the red kangaroo, the common wallaroo, western grey kangaroo (Mayberry *et al.* 2014 ; Pan *et al.* 2012), and the quokka (Gibb *et al.* 1966).

The first cases of *T. gondii* infection in *S. brachyurus*, were documented by Gibb et al. (1966) while retrospectively studying biopsy samples collected from animals on Rottnest Island between 1961 and 1965 (Kakulas 1961 ; Kakulas 1963b ; Kakulas & Adams 1966). Evidence of infection with *T. gondii* (i.e. histopathology, and serology) was observed in 32 of 150 animals studied between 1961 and 1965. Presence of infection was evident in both skeletal and cardiac muscle tissue, the CNS and rarely the kidneys, and was not accompanied by overt signs of the disease (Gibb *et al.* 1966). No gross findings were recorded in Rottnest Island quokkas affected by toxoplasmosis. There was evidence to suggest a possible association between geographical site and *T. gondii* infection, as all positive results were obtained from animals sourced from settled areas on the island. It is likely that infectious oocysts were present on the island given that the domestic cat (*Felis catus*), that was introduced to Rottnest Island by European settlers in the early 1800s (Government of Western Australia 2010), was only successfully eradicated from the island between 2001 and 2002 (Algar, Angus, & Onus 2011).

2.4.5.2 Eimeria spp.

Species belonging to the genus *Eimeria* (> 1,700 described) are a group of enteric coccidian parasites that have a wide range of vertebrate hosts (Bennett *et al.* 2006 ; Hill, Richter, & Power 2012 ; Yang *et al.* 2012). Although exceptions occur (Barker, O'Callaghan, & Beveridge 1988b ; Heckscher *et al.* 1999), host specificity is a characteristic of these parasites, with a single *Eimeria* species usually infecting hosts within a single genus (Austen *et al.* 2014 ; Barker, O'Callaghan, & Beveridge 1988a, 1989 ; Hill, Richter, & Power 2012 ; Yang *et al.* 2012). These parasites have a resilient oocyst stage that allows them to persist in the environment and also makes faecal-oral transmission the most conducive route of transmission (Hill, Richter, & Power 2012). Factors like environmental stresses, malnutrition, wet conditions and overcrowding have been related to outbreaks in captive and free-ranging populations (Vogelnest & Portas 2010). Species identification has traditionally been via oocyst morphology, host specificity and pathology. However, in recent years, molecular characterisation is becoming increasingly important as a tool for *Eimeria* species identification, as eimeriads can be polymorphic (Austen *et al.* 2014 ; Hill, Richter, & Power 2012 ; Yang *et al.* 2012).

Studies of *Eimeria* in wildlife are under-represented, perhaps due to a greater interest on helminths and arthropods (Duszynski & Upton 2001). In Australian marsupials, 56 species of *Eimeria* have been recorded, (Austen *et al.* 2014 ; Duszynski & Upton 2001 ; Hill, Richter, & Power 2012). The genus appears to have a large geographical distribution, having been recorded across all Australian states, in both captive and free-ranging populations (Hill, Richter, & Power 2012 ; Ladds 2009 ; Vogelnest & Portas 2010 ; Yang *et al.* 2012).

Eimeria has been recorded in kangaroos, wallabies, wallaroos, possums, wombats and bandicoots (Austen *et al.* 2014 ; Barker, O'Callaghan, & Beveridge 1988a, 1989 ; Bennett *et al.* 2006 ; Vogelnest & Portas 2010 ; Yang *et al.* 2012), as well as in rat-kangaroos, potoroos and bettongs (Barker, O'Callaghan, & Beveridge 1988a). In WA, western barred bandicoots (*Perameles bougainville*) (Bennett *et al.* 2006) and the southern brown bandicoot (*Isoodon obesulus*) (Bennett & Hobbs 2011) have been found parasitised with *Eimeria*, as well as western grey and red kangaroos, common wallaroos (*Macropus robustus*)(Yang *et al.* 2012) and the quokka. A study by Yang et al (2012) found an overall prevalence of *Eimeria* in macropods to be ~24%.

The disease caused by *Eimeria* in all animals is referred to as coccidiosis, and is an important disease of macropods in captivity (Beveridge 1993 ; Vogelnest & Portas 2010), with young individuals and hand-reared pouch young believed to be particularly susceptible (Vogelnest & Portas 2010). Simultaneous infection with several species of *Eimeria* occurs frequently (Barker, O'Callaghan, & Beveridge 1988a ; Speare 1988b), however, according to Speare (1988b) severe coccidiosis is not a concern in free-ranging populations and this position is supported by the Australian Wildlife Health Network (Australian Wildlife Health Network 2011) and Animal Health Australia. When disease occurs, clinical signs of coccidiosis vary and include signs like abdominal discomfort, lethargy, inappetence, diarrhoea (often haemorrhagic), dehydration, oedema and bruxism (Vogelnest & Portas 2010), though animals can often be asymptomatic (Duszynski & Upton 2001 ; Vogelnest & Portas 2010). While the enteritis produced in macropods may be mild and almost asymptomatic, disease can often lead to rapid death in young animals. Enteric coccidiosis caused by *Eimeria* species generally occurs in young or immunocompromised (e.g. stressed) individuals (Vogelnest & Portas 2010). It manifests as chronic diarrhoea due to moderately high parasitic burdens (Bennett *et al.* 2006). At post-mortem, pathologic lesions associated with coccidial infection usually involve the small intestine and include severe haemorrhagic enteritis with blood throughout the intestinal tract (Potkay 1977 ; Vogelnest & Portas 2010). Other findings consist of

microscopic changes such as acute pseudomembranous inflammation or ulceration, necrosis, oedema, villous atrophy, epithelial cell loss and thickening of the submucosal layer (Ladds 2009 ; Vogelnest & Portas 2010). A hepatic form of coccidiosis is also known to occur in certain species of wallabies and includes findings like granulomatous lesions, fibrosis and biliary duct hyperplasia (Ladds 2009 ; Vogelnest & Portas 2010). Factors that play an important role in the epidemiology of this condition include age (Blyde 1994 ; Munday 1988 ; Speare 1988b), overcrowding (Barker, Harrigan, & Dempster 1972 ; Munday 1978), season (Spratt & Presidente 1978), and starvation (Barker, Harrigan, & Dempster 1972 ; Blyde 1999).

In *S. brachyurus*, *Eimeria* spp. infection has been reported in a few occasions. The work of Barker et al. (1988a) which constitutes the first record of *Eimeria* spp. infection in the quokka, reported three species, all morphologically characterised: *Eimeria setonicis*, *E. volckertzooni* and *E. quokka*. Oocysts were found in faecal samples of 18 out of 19 Rottnest Island animals examined. It was suggested that there was host specificity due to the relative homogeneity in the morphology among *Eimeria* species parasitising the animals (Barker, O'Callaghan, & Beveridge 1988a). A later study by Austen et al. (2014) carried out the molecular characterisation of two of *E. setonicis* and *E. quokka*. They examined sites on Rottnest Island, Bald Island as well as the mainland of WA (Two Peoples Bay: 35 km east of Albany, ~440 km southeast of Perth). Through, microscopy analysis, the overall prevalence of *Eimeria* infection in Rottnest Island animals was 45% (9/20), while a prevalence of 78.3% (18/23) was obtained through PCR. The prevalence of infection in animals captured on the mainland was 62.5% (5/8) by microscopy and PCR methods. Oocysts from *E. volckertzooni* were not isolated in the study (Austen *et al.* 2014). Overall, *E. quokka* appears to be the most prevalent species on Bald Island and the mainland (Austen *et al.* 2014), as well as Rottnest Island (Barker, O'Callaghan, & Beveridge 1988b).

2.4.5.3 Trypanosomes

Trypanosomes are extracellular, flagellated haemoprotozoal parasites that infect all vertebrate classes (Clark 2004 ; Thompson, Godfrey, & Thompson 2014) across a range of habitat types (Smith, Clark*, et al.* 2008). They vary in pathogenicity and are transmitted by vectors such as haematophagous arthropods or leeches and cause disease in their hosts (Botero *et al.* 2013 ; Paparini *et al.* 2011). The epidemiology of trypanosomiasis is influenced by the vector and host relationship. Little is known about the life cycle, pathogenesis and prevalence of trypanosomes in Australia and its offshore islands (Austen *et al.* 2009 ; Bettiol *et al.* 1998 ; McInnes *et al.* 2009 ; Smith, Clark*, et al.* 2008); however

advances in molecular techniques have increased the detection rate of these parasites in the blood of native mammals (Austen *et al.* 2011 ; Paparini *et al.* 2011). Potential vectors of *Trypanosoma* species in Australia may include kangaroo ticks, wombat flea and platypus ticks (Austen *et al.* 2011 ; McInnes *et al.* 2009). Disease may occur particularly when the parasite encounters a naïve host, the presence of concomitant infections or the infected host is exposed to increased levels of stress and becomes immunocompromised (Averis *et al.* 2009 ; Botero *et al.* 2013).

To date, six known *Trypanosoma* species and more than eight genotypes have been recorded in Australian mammals including marsupials such as bettongs (Botero *et al.* 2013 ; Smith, Clark*, et al.* 2008), potoroos (Austen *et al.* 2009), bandicoots (Bettiol *et al.* 1998), koalas (McInnes, Gillett*, et al.* 2011 ; McInnes *et al.* 2009 ; McInnes, Hanger*, et al.* 2011), platypus (Mackerras 1959 ; Noyes *et al.* 1999), chuditch (Smith, Clark*, et al.* 2008), wombat (Noyes *et al.* 1999), flying foxes (*Pteropus* spp.) (Prociv 1987), kangaroos and wallabies (Austen *et al.* 2009 ; Hamilton *et al.* 2005 ; Noyes *et al.* 1999 ; Thompson, Godfrey, & Thompson 2014).

Even though the presence of trypanosomes has not yet been proved to cause disease in free-ranging marsupials (Brock 1999 ; Ladds 2009), their pathogenic characteristics have been proposed to be the main force in the extinction (Breed 2007 ; Wyatt *et al.* 2008) and reduction (Botero *et al.* 2013) of some free-ranging Australian mammal species. Experimental infections in fact, though with exotic trypanosome species, have shown that possums infected with the highly pathogenic *Trypanosoma cruzi*, were affected by an acute trypanosomiasis with a mortality rate of ~60% (Bolliger & Macindoe 1950). In macropods, infection with *Trypanosoma evansi* in agile wallabies and dusky pademelons (*Thylogale brunii*), resulted in 100% mortality, with obvious clinical signs of disease only appearing 24 h before death. These signs included lethargy, tachypnoea, anorexia and ataxia (Reid *et al.* 2001). Other signs of disease that have been reported include fever, fatigue, anaemia and death. Histopathological findings include myocarditis, muscle (skeletal and heart) degeneration, tissue degeneration of the oesophagus and tongue, ulcerative gastritis and enteritis and mononuclear infiltration of the connective tissue in multiple organs (Botero *et al.* 2013 ; Reid *et al.* 2001).

Trypanosomes are also known in *S. brachyurus*. To our knowledge, the first record corresponds to the work of Clark and Spencer (2006) that observed trypanosomes in peripheral blood smears of five quokkas (out of five examined) that were trapped on mainland WA (near Albany). This *Trypanosoma* was not identified, and was not present in

samples from animals (n= 7) trapped on Bald Island. In 2009, Austen et al. (2009) while studying Gilbert's potoroos and quokkas from the same region as that in Clark and Spencer (2006) detected the same *Trypanosoma* in both species, and was named *T. copemani*. The study also established that mixed infections were possible in *S. brachyurus*, with the detection of *T. copemani* genotype A and genotype B (1/3) (Austen *et al.* 2009). In 2011, the tick *Ixodes australiensis* collected from the ears and tails of 15 Bald Island quokkas, was established to be a vector for *T. copemani* genotype A (Austen *et al.* 2011). At the same time, unpublished data suggested that infection with *T. copemani* was not present in quokkas on Rottnest Island, and that animals were not parasitised with ticks but only the biting louse (*Heterodoxus quadriseriatus*) (Austen *et al.* 2011). The most recent record of *Trypanosoma* infection in the quokka is the study by Botero et al. (2013), where *T. copemani* (Clade A) was detected by PCR in one of three mainland animals (carcasses of unknown geographical origin).

The recent association of trypanosomes with the woylie decline (Botero *et al.* 2013 ; Thompson, Godfrey, & Thompson 2014), has led many authors to agree that the impact of trypanosomal disease is especially important to rare and/or endangered marsupials of conservation importance and therefore prudent monitoring should be a priority (Austen *et al.* 2009 ; Averis *et al.* 2009 ; Bettiol *et al.* 1998 ; Botero *et al.* 2013 ; Smith, Clark*, et al.* 2008 ; Thompson, Godfrey, & Thompson 2014). We echo this view in the context of quokka conservation, not just for animals on the mainland and Bald Island where a Clade A trypanosome (*T. copemani*) has been detected, but also for Rottnest Island animals that currently present as an immunologically naïve population, and acute disease is likely to occurred.

2.4.6 ***Neoplasia***

Hubbard et al. (1983) recognised the relative lack of information of neoplasia in zoo animals and emphasised the importance to characterise neoplastic disease in wild animals in light of increasing threats to native species. Tumours that are virally-linked, carcinogen-related or transmissible, impact protected and threatened species via direct or indirect effects on conservation outcomes such as reduction in population sizes or reproductive success (McAloose & Newton 2009). Studies into comparative oncology would provide beneficial information that would help manage disease (Hubbard, Schmidt, & Fletcher 1983), since biopsy tissue samples can be collected from free-ranging species and stored for diagnosis. Although most neoplasms are not infectious, a significant example of effects on a single species is the case of DFTD in the endangered Tasmanian

devil where the high incidence of a transmissible cancer threatens the species with extinction (McAloose & Newton 2009 ; McCallum 2008). It is likely that studies and monitoring of neoplasia in wildlife will not only continue to contribute to better understandings of neoplastic biology but will better inform conservation of species.

Even though neoplastic lesions have been reported in a range of native mammals in Australia, it is difficult to ascertain the prevalence and occurrence of these proliferative lesions considering that proliferative disease can advance quietly, many conditions have non-specific clinical signs, and that animals in the wild with proliferative disease are likely to have short survival rates (Ladds 2009). Archival material from the ARWH has provided the most complete overview of the disease, although the data is marred by limited clinical histories. A review by Ladds (2009) found that between 1974 and 2005, there were reports of 403 neoplasms diagnosed in 402 terrestrial mammals of which 43 cases were in macropods. Out of the 402 cases, the majority were mesenchymal (189) and epithelial (177) neoplasms. Of the 403 neoplasms diagnosed, a large proportion involved the skin and subcutis regions (132) and lymphoreticular organs (87).

In macropods, reviews of archival material as well as of overseas zoo reports (e.g. 1977 ; 2007) found that the majority of macropod neoplastic lesions were epithelial-based with the minority being mesenchymal. Furthermore, most of the reports came from captive macropods, with neoplasia in free-ranging individuals being uncommon, apart from poxvirus-associated papillomas (Ladds 2009 ; Papadimitriou & Ashman 1972 ; Stanley & Mackenzie 1983). Neoplasia has been reported in kangaroos, wallabies, wallaroos and also the quokka (Ladds 2009 ; Vogelnest & Portas 2010).

There have been reports of neoplastic conditions in quokkas since the late 1960s. These reports either appeared in publications concerning marsupial health or were part of the ARWH archived material, which for the most part, relates to captive animals, often with limited background information. The first known report of a neoplastic condition in *S. brachyurus*, dates from 1969, when Appleby mentioned a papilloma of epithelial origin in the tongue of a quokka. Lipomatosis (liposarcoma), of mesenchymal origin (Weiss 1996), was first reported in a three year old female quokka at the University of Western Australia's Zoology Department by Dickson and McNeice (1982). Clinical signs of disease were observed two days prior to death and they included anorexia, loss of balance and coordination with swelling in all four limbs. Post-mortem examination revealed a number of findings that included subcutaneous oedema of the limbs, as well as the abdominal wall with fluid also present in the abdominal cavity (Dickson & McNeice 1982). Present in the

mesentery and peri-renal area, were lumps (length ≤8 cm, diameter ~4 cm) of creamy white material that were flat, smooth and avascular. Histopathology studies identified the masses as fat, and also found necrosis of a number of whole liver lobules that was suggested to be associated with vascular occlusion. A diagnosis of abdominal lipomatosis (i.e. liposarcoma) was made and there was no evidence of metastases in any organ (Dickson & McNeice 1982). Two reports, one of a mammary gland adenocarcinoma and another of an infiltrative pancreatic lipoma were described by Ladds (2009) and Vogelnest and Portas (2010) respectively, but there is no other information available concerning these cases. A dermal lymphosarcoma has been previously reported in *S. brachyurus* (Canfield, 1990 in Vogelnest & Portas 2010), however, we believe this record is incorrect (in Vogelnest and Portas), as the only proliferative lesion in the quokka reported by Canfield et al. (1990a) in their review on spontaneous proliferations in Australian marsupials, corresponds to a papilloma of the tail attributed to a poxvirus, citing the work of Papadimitriou and Ashman (1972).

Other reports of neoplasms in *S. brachyurus* are listed on the Online Registry of the ARWH (Table 2-4,- as of July 2015). Metastasis was observed in only one case. With the exception of the thyroid and biliary adenomas, all lesions were considered a primary diagnosis.

Table 2-4 Neoplastic-proliferative lesions reported in *S. brachyurus* as final primary or secondary diagnosis in the Online Registry of the ARWH.

Type of lesion	Year	Sex	Age	Origin	Clinical History	Post-mortem and histopathology findings
Infiltrative liposarcoma	2000	male	adult	Rottnest I.	Weight loss, lethargy, vomiting, irregular and hard masses in dorsal and ventral abdomen, only gas present in the GI tract	Firm, irregular, white mass of ~3cm diameter replacing the pancreas, multifocal granulomatous pneumonia, focal thoracic abscess. Positive to *C. gattii* and *Streptococcus viridans* group 1
Mammary adenocarcinoma	2004	female	adult	Rottnest I.	Weight loss	Metastasis to mesentery, liver and inguinal lymphnodes, diffuse lung congestion, acute tubulitis and tubular cysts, and a cystic ovary
Pericloacal carcinoma and Thyroid adenoma	2006	female	adult	Rottnest I.	Sudden cloacal prolapse that had ulcerated and involved the urethra, cataracts; there was a history of hepatopathy and myopathy	Mineralisation and saponification of mesenteric fat deposits, cystic endometrial hyperplasia, membranous glomerulopathy and suppurative nephritis
Biliary adenoma	2010	female	adult	Rottnest I.	-	Not a primary diagnosis
Mammary adenocarcinoma	2012	female	adult	Rottnest I.	Anorexia, diarrhoea and lethargy, large firm mammary masses one on each side. Lesions had spread to the abdomen three weeks later	Necrosis of the adipose tissue, pulmonary oedema, multifocal lymphoid hyperplasia, multifocal membranous glomerulonephritis, as well as multifocal myocardial degeneration

2.5 Conclusion

Endemic to WA, the quokka is a resilient species that has persisted throughout millennia despite pressures such as hunting, predation, changes in fire regimes and at least five events of disease, once of which caused a drastic decline that might have began as early as 1901. Some populations on the mainland appear to be increasing, possibly in response to the mitigation of certain pressures (especially control of European red fox numbers) but the species is still considered by local and international conservation agencies, to be 'vulnerable'. The species faces a drastic population fragmentation across the entire range, and in conjunction with a genetic bottleneck of the island subpopulations (of which Rottnest Island represents 40-50% of the estimated total numbers for the species), the

increasing pressures of climate (e.g. longer droughts), disease and the continuous expansion of human settlements and industry, mean the future of this species is uncertain.

Like other ecological factors, it has been shown that disease plays an important and dynamic role in the persistence of wildlife populations and can push species to extinction. Because of the risk of potential catastrophic effects of disease on the conservation status of the quokka, proper long-term management of the species requires a holistic approach that recognises the role and risks of pathogens. Consequently, better understandings of pathogens and parasites in *S. brachyurus* is critical. In this sense, we believe this review, in conjunction with the following chapters, provide a comprehensive study of the diseases and pathogens in the quokka that can be integrated into current and future species-survival plans.

3. General Methods

STATEMENT OF AUTHOR CONTRIBUTION

P. Martinez-Perez: designed the general scope and structure of the chapter, wrote the grant applications to obtain the operational funds necessary to carry out the project, purchased and obtained all necessary reagents and equipment, performed all field procedures [i.e. trapping on Rottnest Island, anaesthesia, physical examination, sample collection, sample processing (serum separation, blood smears), microchipping, ear tagging, ear notching], carried out blood smear assessment, and wrote the chapter.

P.A. Fleming: assisted with the connections necessary to obtain animals on the mainland, advised, assisted and provided editorial comments to grant applications, and provided editorial comments to version of the chapter from draft to final version.

S. Dundas and K. Bain: carried out trapping on the mainland.

M. Bennet and C. Monaghan: overviewed all veterinary procedures (on paper), provided field assistance (two occasions), provided editorial comments to versions of grant applications, and provided editorial comments to versions of the chapter from draft to final version.

T.H. Hyndman: provided editorial comments to versions of the chapter from draft to final version.

Murdoch University Veterinary Hospital: carried out the haematology (except blood smear assessment) and blood chemistry for all blood samples used in the experimental chapters.

3.1 Study period and study sites

Free-ranging quokkas were trapped on Rottnest Island and the mainland during 2010 and 2011 (Figure 3-1). Some limitations when trapping on the mainland were present: (i) animal movement is much higher on the mainland as the animals have more land to roam, therefore population density is much less, which had a direct impact on trapping success when compared to Rottnest Island; and (ii) as this project did not performed its own trapping, the animals that could be included were determined by those projects capturing quokkas for other research purposes. This resulted in a rather smaller than expected

sample size, and mostly adult males being sampled. With few exceptions, animals were not recaptured. Hence, animals were released immediately after being trapped once it was determined that they have already been captured and sampled.

All medical procedures were carried out under Murdoch University Animal Ethics Committee permit No. W2309/10, and Department of Environment and Conservation (now known as the Department of Parks and Wildlife) Regulations 4 No. CE002891, and 17 No. SF007550.

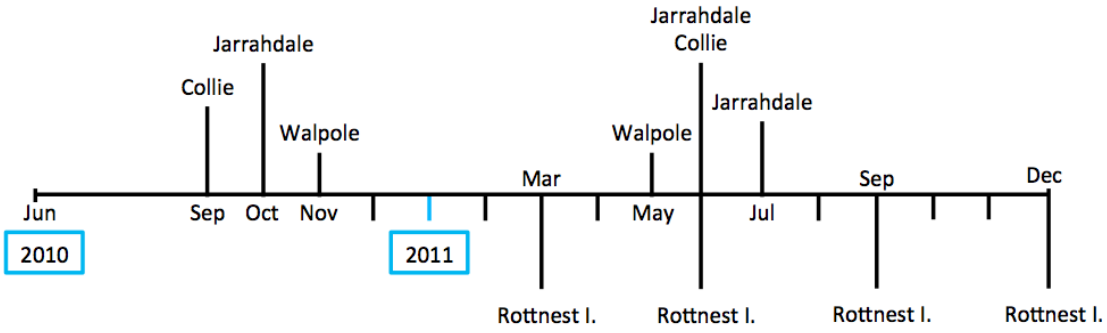

Figure 3-1 Times of the year in which trapping of *S. brachyurus* was carried out on the mainland and on Rottnest Island

3.1.1 *Rottnest Island*

Rottnest Island is located approximately 32 km west of Perth and 19 km west of Fremantle. The island is 11 km long, 4.5 km at its widest point and has an area of 1,900 ha (Government of Western Australia 2010). It has been estimated that Rottnest Island separated from the mainland by rising sea levels about 7,000 years ago (Glenister, Hassell, & Kneebone 1959) and is the largest Quaternary limestone island off the coast of Western Australia (WA) (Glenister, Hassell, & Kneebone 1959 ; Government of Western Australia 2010). Rottnest is different from the other more than 500 islands off the coast of WA, for two main reasons. It is the only island with extensive areas of saline inland waters, and it is a Class A Reserve to be used for "Public Recreation" (Saunders & De Rebeira 2009).

Rottnest Island has a temperate climate (Australian Bureau of Meteorology 2012). The mean monthly temperatures range annually from 15.6 – 22.1°C. Mean annual rainfall is 583.6 mm. The vegetation coverage of Rottnest Island has changed considerably in the past. Native pines (e.g. *Callitris preissii*), and tea-trees (e.g. *Melaleuca lanceolatta*) were

abundant (Dunnet 1962 ; Stevenson 2011) before European settlement. Today, exotic species like eucalypts (e.g. *Eucalyptus platypus*), are dominant in urban areas while open areas are characterised by variable speargrass (*Stipa variabilis*) and prickle lily (*Acanthocarpus preissii*). Rottnest Island lacks natural streams of fresh water, and the only naturally occurring surface water is found in lakes and is highly saline.

Animals were trapped on Rottnest Island (~32 km west of Perth) at six locations (i.e. Barkers Swamp, Kingston, Parker Point, Settlement, Serpentine and West End) (Figure 3-2). Exact geographical coordinates for each trapping site are given in Table 3-1.

Figure 3-2 Map of Rottnest Island with the locations where *S. brachyurus* were trapped. Map data ©2016 Google, Data SIO, NOOA, U.S. Navy, NGA, GEBCO.

Table 3-1 Location details of the multiple sites where *S. brachyurus* were captured on Rottnest Island

Sites	Latitude	Longitude	Environment class
Serpentine	32° 0' 27.1362" S	115° 31' 21.234" E	Less disturbed [a]
Parker Point	32° 1' 21.2232" S	115° 31' 39.4998" E	Less disturbed
Barkers Swamp	32° 0' 5.6658" S	115° 30' 19.8072" E	Less disturbed
Kingston	32° 0' 16.0776" S	115° 33' 15.246" E	Disturbed [b]
West End	32° 1' 13.4754" S	115° 27' 29.3934" E	Undisturbed [c]
Settlement	31° 59' 47.238" S	115° 32' 23.8734" E	Disturbed

[a] defined as those that had less human presence, infrastructure was still present, but access to unconventional food sources was much lower

[b] defined as those in which human interaction was relatively constant, infrastructure was more common, there was a high visitation flow, and alternative food was readily available to the quokkas from restaurants and visitors

[c] characterised for practically not having any infrastructure, for having thicker vegetation cover making it very difficult to visitors to venture in, and sources of unconventional food were much lower or non-existent

3.1.2 *Mainland*

On the mainland, quokkas were trapped near Jarrahdale (~45 km south-east of Perth), Collie (~213 km south of Perth), and near Walpole (~411 km south south-east of Perth) (Figure 3-3). Exact geographical coordinates for each trapping site are given in Table 3-2.

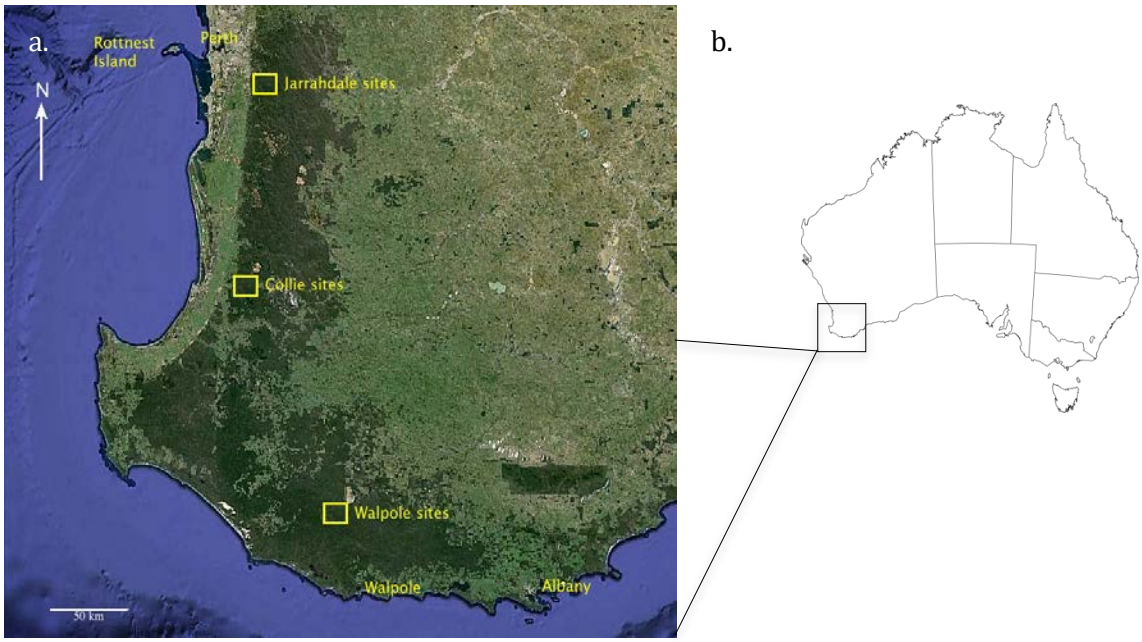

Figure 3-3 (a) Close up map of the south-west of Western Australia with the mainland locations where *S. brachyurus* were trapped. Rottnest Island, Perth, Walpole and Albany are provided in this map only as a point of reference. (b) Location of the southwest of Western Australia in Australia. Map data ©2016 Google Landsat, Data SIO, NOOA, U.S. Navy, NGA, GEBCO.

Table 3-2 Location details of the northern and southern sites where *S. brachyurus* were captured on the mainland of the south-west of Western Australia.

Sub-populations	General Sites	Specific Sites	Latitude	Longitude
Northern Jarrah	Northern sites			
	Jarrahdale	Thirty One Mile	32° 15' 55.872" S	116° 10' 24.0234" E
		Mile	32° 13' 6.636" S	116° 8' 0.024" E
		Midgegoroo	32° 11' 2.04" S	116° 6' 27.468" E
		Rosella	32° 16' 5.628" S	116° 4' 42.708" E
		Balmoral	32° 20' 28.572" S	116° 4' 46.3434" E
		Chandler	32° 17' 49.7034" S	116° 7' 51.132" E
Central Jarrah	Collie	Hamilton	33° 15' 48.528" S	116° 2' 2.04" E
		Victor	33° 16' 10.2" S	116° 0' 59.2554" E
		Gervasse	33° 21' 32.1114" S	115° 55' 14.6994" E
Southern Forest	Southern site			
	Walpole	Thompson Rd.	34° 39' 35.5998" S	116° 42' 14.7456" E
		Martin Rd.	34° 37' 34.863" S	116° 29' 57.8574" E

3.2 Trapping protocols

Animal capture on Rottnest Island commenced one hour before sunset and extended into the night to minimise disturbance to and by the public. *Setonix brachyurus* were captured either by trapping or hand capture. For hand capture, animals were quietly approached and then captured by restraining the animal at its tail base (Vogelnest & Portas 2010). No animals were ever chased and all animal handling was kept to a minimum. Alternatively, six Thomas traps® (Sheffield Wire Products, Sheffield Rd Welshpool, Perth WA) (Department of Environment and Conservation Standard Operating Procedure SOP 9.5 for the capture of small to medium sized macropods, Freegard & Richter 2009b) were deployed at each of two sites per night after sunset, with trap clearing and animal processing extending into the night. Traps were located on the verge of the road for ease of access and to minimise disturbance to the vegetation and were baited with a small ball (i.e. 2 cm diameter) of freshly prepared 'universal bait' (peanut butter with plain oats). During summer, fresh water was also provided. Traps were checked and cleared every hour at each site. Upon capture, animals were individually transferred to a calico bag and transported within 5 – 12 min to an indoor processing station, for weather protection and sufficient lighting.

Animals on the mainland were sourced from several concurrent projects that involved trapping *S. brachyurus*; consequently, trapping protocols varied somewhat between sites. Trapping was not carried out on days where the weather forecast predicted temperatures over 35˚C. Traps were generally deployed along water systems and baited with apples and were cleared the following morning. Animals were trapped at the northern sites with Sheffield traps (Sheffield Wire Products, Sheffield Rd Welshpool, Perth WA) (Department of Environment and Conservation Standard Operating Procedure SOP 9.2: Cage traps for live capture of terrestrial vertebrates, Freegard & Richter 2009a). At the southern sites, animals were captured using Thomas traps. For both northern and southern sites, traps were each covered with a hessian and a plastic bag in order to protect the animals from light/disturbance and the weather. Upon capture, quokkas were individually transferred to a hessian bag and carried to the point of processing. It is worth noting that prior to all procedures ran by this project (i.e. physical restraint, general anaesthesia, physical examination and biological sample collection), a series of biometrical measurements were carried out on all animals by crewmembers of the projects carrying out the trapping. Animals were weighed while in the bag with a digital fishing scale (± 0.5 g), upon which the weight of the bag was substracted.

Anaesthetic equipment, calico and hessian bags were disinfected with F10SCXD disinfectant (Health & Hygiene (Pty) Ltd. Sunninghill, South Africa) between field trips. Calico bags were additionally autoclaved at Murdoch University Veterinary Hospital. Restraining bags were never transferred between Rottnest Island and mainland sites.

3.3 Anaesthesia, physical examination and general procedures

Prior to sample collection, all animals were anaesthetised. At the processing station, quokkas were physically restrained while in their bag for anaesthesia induction. The nose of the animal was exposed from the bag and the animal was induced with 5% isoflurane I.S.O.® (Veterinary Companies of Australia, Kings Park, NSW) delivered in 100% medical oxygen via a Darvall® (Advanced Anaesthesia Specialists, Gladesville, NSW) facemask connected to a non-rebreathing Bains Circuit® (VetQuip, Castle Hill, NSW), with an oxygen flow rate of 2.5L/minute. Anaesthesia was carried out using a Stinger® Streamline anaesthetic machine (Advance Anaesthesia Specialists, Gladesville, NSW). Anaesthetic status was assessed through loss of general muscle tone, presence or absence of corneal reflex and withdrawal response measured via toe pinch (usually 2 – 3 min post induction) before the animal was removed from of the bag and laid in right lateral recumbency for physical examination. Once anaesthesia was induced, the concentration and flow rate of isoflurane was reduced to 2-3% at 2L/min. Respiratory rate and heart rate were monitored at least twice during the extent of the procedure, which commonly took between 8 –12 min. Cloacal temperature was monitored and bubble wrap and towels were used to maintain cloacal temperature between 36.5 °C and 38.5 ˚C (Bartholomew 1956).

The physical exam was organised by anatomical regions, and findings were recorded in a clinical examination sheet designed by this project (Figure 3-4). Physical examination was carried out starting with the tail and finishing with the head, initially in left lateral recumbency and subsequently in right lateral recumbency. The severity and chronicity of any observed lesions was recorded. Body condition was first assessed according to a subjective scale (Table 3-3) followed by hydration status (Table 3-4) and assessment of the colour and texture of mucous membranes (Table 3-5). External parasites (i.e. ticks, lice and fleas) were counted, the anatomical area where they were spotted, and their developmental stage (i.e. larva, nymph or adult) was recorded. The scoring schemes used in the physical examination of quokkas (i.e. Tables 3-3, 3-4 and 3-5) were designed by this project, and used general principles and techniques available in the wildlife and domestic

animals veterinary literature (Gamble 2004 ; Smith 2008 ; Vogelnest 2015). Although the physical findings listed in Table 3-6 are results of this project, these are listed here as part of the methodology as their definitions were a priori.

QUOKKA FIELD HEALTH CHECK FORM

Quokka Health status and Diseases Project. Murdoch University.

Site Name:_____ Date/Time: _____ Species: Setonix brachyurus ID: _____

Handler: _____ Recorder: _____ Weight:_____ Body Cond:_____ Gender: ____ Age: YA – SA – A

Anaesthesia Notes

Induction Time		Start Body T°		MM		Heat Pad		Weather	Temp:	Humidity:
Starting Time		Mid Body T°		CRT						
Ending Time		End Body T°								

	LS	RS	LS	RS			Ticks		Lice		Fleas	
Anatomical Region	**Descriptor / Severity Code**		**Time Scale**		**Comments**	**Ph**	**Cnt**	**Stg**	**Cnt**	**Stg**	**Cnt**	**Stg**
Mentation	BAR, Coma, Depressed, Hyper-excitability, Stupor, QAR (please mark as indicated)											
Hydration	0 – 1 – 2 – 3 – 4 (please mark as indicated)											
Head												
Eyes												
Ears												
Nose												
Neck												
F. Limbs												
Chest/Resp												
Abdomen												
Pouch												
Testes												
Cloaca												
Back/Shoulder												
H. Limbs												
Rump												
Tail												

Samples (please circle) EDTA – Lit. Heparin - Serum - Faecal (bag/animal) - Nasal Swab - Urine - Blood smear - Biopsy (Abnormal mass) - Ear notch (Genetics)

Other comments: _____

Figure 3-4 Clinical examination sheet used to record basic health data from *S. brachyurus*. MM= mucous membranes, CRT= capillary refill time, LS= left hand side, RS= right hand side, Ph= photo, Cnt= count, Stg= stage of development, BAR= bright, alert, responsive; QAR= quiet, alert, responsive.

Table 3-3 Semi quantitative body condition scores used in *S. brachyurus*, and the features observed on the animal that defined each of the scores.

Body condition scores	Description	Features observed that defined each score
0	Emaciation	Scapular spine and dorsolateral and lateral processes of the first two coccygeal vertebrae are prominent. There is an obvious concavity of skin and muscle around these vertebrae).
1	Poor	Scapular spine and dorsolateral and lateral processes of the first two coccygeal vertebrae are still palpable and visible. The concavity of skin and muscle around them is less.
2	Optimal	Scapular spine and dorsolateral and lateral processes of the first two coccygeal vertebrae are barely palpable. Concave appearance of the skin and muscle around the bony prominence is not present.
3	Overweight	Scapular spine and dorsolateral and lateral processes of the first two coccygeal vertebrae are not palpable. There is a slight convex appearance of the skin and muscle around the bony prominence.
4	Obese	Scapular spine and dorsolateral and lateral processes of the first two coccygeal vertebrae are not palpable. Marked convex appearance of the skin and muscle around the bony prominence.

Table 3-4 Semi quantitative assessment of hydration status of *S. brachyurus*, and a description of how they were determined.

Value	Dehydration status (expressed as % of body weight)	Description
0	0-5%	No detectable abnormalities, skin under neutral tension (e.g. the skin over the thorax) returns to normal position after tenting in <2 sec, CRT <2 sec, enophthalmos (sunken eyes) absent, mucous membranes pink and moist.
1	5-8%	2-4 sec delay of the thoracic skin returning to normal position, increase in CRT to 2-3 sec, enophthalmos slightly present, mucous membranes slightly dry or tacky.
2	8-10%	4-8 sec delay of thoracic skin returning to normal position, increase in CRT to 3-4 sec, enophthalmos more obvious, mucous membranes dry and slightly tacky.
3	10-12%	Greater than 8 sec delay of thoracic skin returning to normal position, CRT increased to beyond 4 sec, eyes very sunken, dry mucous membranes, animal is depressed, signs of shock may be present (e.g. rapid/weak pulse, cold extremities).

Table 3-5 Mucous membrane appearance in *S. brachyurus* and the possible implications of each appearance.

Mucous Membranes Appearance	Possible Implications
Pink/Pale pink	Implies adequate perfusion and oxygenation of peripheral tissues. Some vasoconstriction due to low environmental temperature or stress
Pale White	Anaemia, poor perfusion, vasoconstriction (secondary to blood loss, shock, decreased peripheral blood flow)
Blue	Inadequate oxygenation (hypoxemia)
Brick Red	Increased perfusion, vasodilation (secondary to early shock, sepsis, fever, systemic inflammatory response syndrome)
Yellow	Bilirubin accumulation (liver or biliary disorder and/or haemolysis)
Brown	Methaemoglobinaemia (e.g. intravascular haemolysis)
Petechial haemorrhage	Coagulation disorder (platelet disorder, coagulation factor deficiencies)

Table 3-6 List of physical findings recorded in quokkas on Rottnest Island and the mainland of Western Australia, with their corresponding description.

Physical findings	Description
Abnormal mentation	A quokka that did not struggle at the moment in which was hand-picked, that was obtunded, unresponsive to human handling, that did not struggle and appeared unresponsive during GA induction (e.g. dull, stuporous, comatose)
Abnormal CRT	That in which blood would return to the tissue in 2 or more seconds after putting pressure on the gums
Erosion	Single or multiple lesions on the skin in which part or all of the epidermis has been lost. Depending on the severity it may be accompanied by bleeding of the outermost layer of the dermis
Cloacal discharge	Abnormal cloacal discharge was that of a colour typically associated with bleeding (red), infection (mucopurulent)
Cloudy eye	An eye with a diffuse or focalised opacity on the cornea that appears in the front of the eye as white or somewhat blue in colour
Conjunctival hyperaemia	The injection of the conjunctival vessels giving the conjunctiva a pink/red colour, with ocular discharge usually mucoid of mucopurulent, and epiphora (determined by the fur directly underneath the medial canthus being wet)
Ear notches	Single or multiple lesions in which the ear cartilage has lost entire sections of it, making it have an incomplete edge
Flaky skin	Single or multiple skin lesions in which 'flakes' represent desquamated layers of the stratum corneum
Fractures	Acute fractures were those in which displacement of bone fragments was present, while chronic fractures were those bone lesions characterised by the presence of a callus, a loss of external typical appearance, and the absence of obvious signs of inflammation
Fur loss	Single or multiple patches of exposed skin that have lost their corresponding covering fur
Skin thickening	Thickening and hardening of skin characterised by exaggeration of superficial skin markings. Usually accompanied by hyperpigmentation
Ulcers	Single or multiple discontinuities of the epidermis with exposure of the dermis, characterised by the presence of a crater and some exudate
Wheezes	Respiratory 'musical' tones that occur during inspiration and expiration as a result of a decrease in the airway lumen
Presence of external parasites	External parasites where either present or absent, type, numbers and the anatomical region where found were recorded

Since obtaining data from different animals at a single point in time was this entire project required, animal recapture was avoided. For this, animals had to be identified. Identification of animals on the mainland was carried out by concurrent projects trapping quokkas. On Rottnest Island, quokkas were microchipped and ear tagged. Animals were microchipped with FDX B Transponders (AllFlex Australia PTY Ltd. Capalaba, Queensland, Australia) that were read with the RS200 Series Compact Reader (AllFlex Australia PTY Ltd. Capalaba, Queensland, Australia). Microchips were implanted subcutaneously between the scapula in accordance with Department of Environment and Conservation Standard Operating Procedure 12.1. (Richter & Freegard 2009a) and veterinary guidelines established by the Australian Veterinary Association (Australian Veterinary Association 2013) were followed. Briefly, the skin between the scapulas was cleaned with a mixture of chlorhexidine and alcohol. Subsequently, the microchip was implanted in a cranio-caudal orientation in to the subcutaneous space, and the point of entry was sutured with tissue adhesive (Vetbond™ 3M™, MN, USA). Ear tagging was done in accordance to the

Department of Environment and Conservation standard operating procedure 12.3: Semi-permanent marking of animals using ear tags (Richter & Freegard 2009b). Figure 3-5 shows the location of the ear tags in females and males. Animals were tagged with Monel self-piercing ear tags size 3 (#1005-3) from the National Band & Tag Co. Newport, USA. Any bleeding that occurred was controlled by applying pressure on the area with an sterile gauze until clotting had occurred. Similarly to microchipping, the area for the ear tag was cleaned with a mixture of chlorhexidine and alcohol.

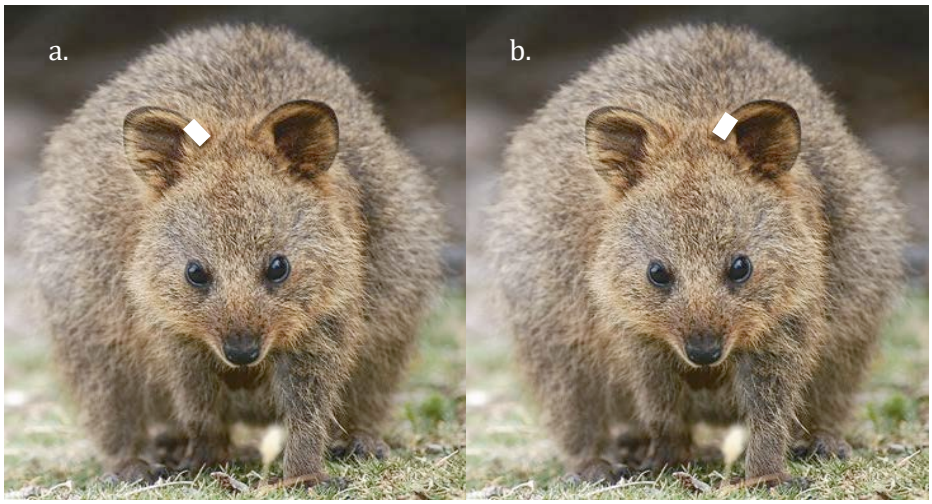

Figure 3-5 Position of the ear tags indicated by a white rectangle, in females (a) and males (b) *S. brachyurus*.

Pouch taping was always performed on the adult females that had small joeys (i.e. hairless and/or lightly furred). The procedure followed the guidelines established by the Department of Environment and Conservation in their Standard Operating Procedure 14.1: *Care of evicted pouch young* (Freegard & Richter 2009c). Briefly, the calico bag was initially checked for the presence of a furred joey that may have been ejected. If present, this joey was taken out of the bag before initiating the anaesthetic induction of the adult female, and kept warm temporarily against an operator's body. Subsequently, having the female under anaesthesia, the pouch was inspected for the presence of a hairless infant. If not present, the furred joey was delicately inserted into the pouch head first allowing him to crawl in by itself. With the joey back into its pouch, the fur around (i.e. 4 cm) the pouch was clipped to ensure adequate adhesion of the tape (Fixomull® stretch, BSN Medical, Charlotte, USA). Having both opposing edges of the pouch together, the tape was positioned longitudinally so there was no contact with any mucosal area of the pouch. With the joey in position and the pouch taped, the physical examination procedure resumed.

Upon completion of the physical examination and sample collection, the Isoflurane vaporiser was turned off, the animal was put once more into its own bag, the anaesthetic circuit was flushed to remove remaining anaesthetic gaseous agent without having the animal connected, and the quokka remained with pure (100%) oxygen at a flow rate of 2L/min until withdrawal responses returned, and the swallowing reflex was present. On Rottnest Island, animals were next to each other within their own bags to favour heat exchange and minimise loss of body temperature. A repeated inspection of each animal during recovery was performed. Heat packs (Livingston International Pty Ltd, New South Wales, Australia) were used on several occasions to help prevent a drop in core body temperature. Once mentally alert and ambulatory (usually about 5 – 10 min following anaesthesia), animals were released back to their original trap site.

3.4 Sample collection, handling and studies

3.4.1 *Faeces*

Faecal samples for the isolation of *Salmonella* were obtained by rectal palpation using examination gloves. Faecal pellets were used instead of rectal swabs as this type of sample produced better results when doing isolations from samples with a low number of *Salmonella* (Hart, Bradshaw, & Iveson 1985). The external area around the cloaca was cleaned with a 1:1 mixture of chlorhexidine gluconate and 70% ethanol (care was taken to prevent the disinfectant from getting into contact with the faeces) and any dirt and faecal material was wiped off immediately before rectal palpation. Some animals spontaneously defecated after rectal palpation, however, even though the pellet was not collected directly from the rectum, the sample was still considered viable. In any case, the main condition any sample should meet to be considered valid was to not have come in contact with any other surface than the cloacal lining and an examination glove. In no cases were faecal samples taken from cotton/hessian bags, or traps. Samples were then placed into 5 mL polycarbonate yellow cap sterile tubes (SARSTEDT Aktiengeseilschaft & Co. Germany) and stored at 4˚C until processing. Helminth and protozoan parasites were detected using the zinc sulphate flotation technique. Briefly, the flotation solution was prepared by diluting 330 g of zinc sulphate in 1 L of distilled water. Faecal samples (~5 g) were mixed thoroughly with zinc sulphate solution in 10 mL polycarbonate yellow cap sterile tubes (SARSTEDT Aktiengeseilschaft & Co. Germany). The tubes were filled with the flotation solution until this had created a convex appearance over the edge of the tube, then a glass coverslip was placed on top and the tubes were centrifuged at 2,500 g for 7 min. Lastly, coverslips were removed from the 10 mL polycarbonate tubes and place on a microscope

slide to be then examined using an Olympus BX50F4 (Olympus Optical Co, Ltd. Japan) light microscope for oocysts and parasite eggs. Oocysts and nematode eggs were counted in 50 high power fields (hpf). For oocyst sporulation, faecal samples were mixed thoroughly with 2% potassium dichromate (w/v) solution in a Petri dish that was left incubating at room temperature in the dark. Aliquots of 20 µL were then collected every three days and placed on a slide with a coverslip and check for sporulation under light microscopy. This volume was obtained by collecting smaller volumes from different areas of the Petri dish. If sporulation was observed, morphologies and dimensions were taken with ImageJ v. 1.49d (Schneider, Rasband, & Eliceiri 2012) for further speciation.

3.4.2 *Blood*

Blood samples were collected from the lateral left or right tail veins. Skin was prepared by clipping the venipuncture site with WAHL® clippers (Wahl Clipper Corporation and Unity Agencies, Victoria, 3180, Australia), which was subsequently disinfected with a 50/50 chlorhexidine gluconate and 70% ethanol solution. Blood was obtained using Safety-Lok™ BD Vacutainer® 25G x ¾" and 23G x ¾" (Becton, Dickinson and Company, NJ, USA) with Slip Tip 3 mL syringes (Becton, Dickinson and Company, NJ, USA). Blood for haematology analyses (~0.5 mL) was collected in 600 µL BD Microtainer® tubes with potassium (K_2) ethylene diamine tetraacetic acid (EDTA) anticoagulant (Becton, Dickinson and Company, NJ, USA), while samples for blood chemistry (~1.0 mL) analyses were collected in 1.3 mL Micro Tubes with 35 I.U. of Lithium Heparin / 1 mL of blood (SARSTEDT, Aktiengesellschaft & Co. Nümbrecht, Germany). These two blood samples were mixed gently upon collection and stored at 4 ˚C for further processing. Blood for serology studies (~3 mL) was collected in 4 mL Serum BD Vacutainer® (Becton, Dickinson and Company, NJ, USA) and left standing to clot for 6h. Tubes were then centrifuged and serum was carefully withdrawn without disruption of the clot layer. Serum was then stored at -20 ˚C for further processing.

Blood in EDTA and Lithium Heparin were processed within the first 60 h with the majority of the samples been processed by 48 h post collection. EDTA and Lithium Heparin samples were sent to the Clinical Pathology service of the Murdoch University Veterinary Hospital. A complete blood count was obtained with an ADVIA-120® automated haematology analyser (Bayer Diagnostics Division) and multi-species software using the default setting (canine), while an RX Daytona™ automatic biochemistry analyser (Randox Laboratories) was used for blood chemistry. Table 3-7 shows the haematological and blood chemistry variables typically analysed.

Table 3-7 List of haematological and blood chemistry analytes measured on whole blood obtained from mainland and Rottnest Island *S. brachyurus*.

Analyte	Acronym
Haematology	
White Blood Cell Count	WBC
Red Blood Cell Concentration	RBC
Haemoglobin Concentration	HGB
Packed Cell Volume	PCV
Mean Corpuscular Volume	MCV
Corpuscular Haemoglobin Concentration Mean	CHCM
Platelet Concentration	PLT
Neutrophils	NEUT
Lymphocytes	LYMPH
Monocytes	MONO
Eosinophils	EOS
Basophils	BASO
Blood Chemistry	
Alkaline Phosphatase	ALP
Alanine Aminotransferase	ALT
Aspartate Aminotransferase	AST
Creatine Kinase	CK
Gamma-glutamyl Transferase	GGT
Total Protein	TP
Albumin	ALB
Globulin	GLOB
Calcium	CALC
Phosphorus	PHOSP
Cholesterol	CHOL
Total Bilirubin	BILT
Glucose	GLUC
Creatinine	CREAT
Urea	UREA
Vitamin E	Vit. E

A blood smear was made using the spreader slide technique within a couple of hours after blood collection. The smear was then stained with Hema-tek® Slide Stainer and Hema-tek® Wright's Giemsa Stain (Ames Company, Miles Laboratories). Smears were then assessed using light microscopy to determine the differential leukocyte count (200 leukocytes), polychromatophilic erythrocyte count (1,000 erythrocytes), and leukocyte and erythrocyte morphology. Presence and numbers of microfilariae were obtained by examining the complete blood smear at x40 magnification but anatomical characteristics of microfilariae were recorded at x400 or x1000 magnification. The presence of intraerythrocytic organisms was assessed by light microscopy, examining 100 fields within the monolayer and feathered regions of the blood smear combined, first at x400 magnification, and subsequently at x1000 magnification and by nPCR (see Chapter 7, section 7.3.1.1.). The ADVIA white blood cell count was corrected by subtracting the polychromatophilic erythrocytes count. This value was then used in subsequent analyses. Serum samples of approximately 300 µL were shipped in dry ice to Professor Colin Wilks at Melbourne University, School of Veterinary Medicine for Macropodid herpesvirus Type

I and II serology, and approximately 100 μL to the Department of Primary Industries, Animal Health Laboratories in Tasmania, for *Toxoplasma gondii* IgG testing via the MAT.

Overall, the range of organisms studied as well as the tests to which this study had access to, were limited primarily by costs.

4. *Salmonella* and the health of *S. brachyurus* from Rottnest Island and the mainland of south-west Western Australia

STATEMENT OF AUTHOR CONTRIBUTION

P. Martinez-Perez: designed the general scope and structure of the chapter, initiated the research, carried out all fieldwork procedures, carried out all laboratory procedures for the isolation and preliminary identification of *Salmonella* isolates, performed blood smear assessment, entered and analysed the data, and wrote the chapter.

P.A. Fleming: advised and assisted with statistical analyses, and provided editorial comments to versions of the chapter from draft to final versions.

T.H. Hyndman: provided editorial comments to versions of the chapter from draft to final versions.

K. Townsend: provided training in culturing techniques.

PathWest: carried out serotyping and antimicrobial sensitivity tests.

4.1 Abstract

Salmonella are Gram-negative, motile and facultative anaerobe bacteria with a worldwide distribution that contaminates multiple substrates (vegetation, food, soil and water) and inhabits the gastrointestinal tract of birds, reptiles and mammals, including humans. Clinical disease in animals is usually triggered by stress and presents in various forms ranging from subclinical carriers to acute endotoxemia and sepsis. Current data on the prevalence of *Salmonella* infection in Rottnest Island and mainland quokkas is out dated by approximately 30 years. Additionally, previous studies of *Salmonella* in quokkas did not perform physical examinations or diagnostic tests in order to explore the potential clinicopathological implications of such infection. We screened 92 quokkas (*Setonix brachyurus*) from Rottnest Island (n= 71) and populations on the mainland (n= 21) for *Salmonella*, and determined a prevalence of 47.9% (95% CI 36.5-59.4) and 4.8% (CI 0.8-22.7), respectively. A total of 16 serovars were identified in 37 isolates, of which five new serovars not previously described in the quokka, are reported. *Salmonella* appeared to have a subtle effect on the haematology (HMT) and blood chemistry (BLC) of quokkas on

Rottnest Island, while no effect was observed on peripheral blood cell morphologies (PBCM). Animals with circulating microfilariae were 3.88 (CI 1.31-11.50, $p < 0.05$) times more probable of being infected with *Salmonella* spp.

4.2 Introduction

Salmonella sp. are rod-shaped, Gram-negative, motile and facultative anaerobes classified in the family Enterobacteriaceae (Nataro *et al.* 2011). These organisms have a worldwide distribution, contaminating vegetation, food, soil and water, and inhabit the gastrointestinal tract of birds, reptiles and mammals, including humans (Quinn *et al.* 1994 ; Quinn *et al.* 2011) (Table 4-1).

Table 4-1 *Salmonella species, subspecies, serovars and their most common habitats* (Brenner *et al.* 2000)

Salmonella species, subspecies (group)	Regular habitat
S. enterica subsp. *enterica*	Homoethermic animals
S. enterica subsp. *salamae* (II)	Ectothermic animals and the environment
S. enterica subsp. *arizonae* (IIIa)	Ectothermic animals and the environment
S. enterica subsp. *diarizonae* (IIIb)	Ectothermic animals and the environment
S. enterica subsp. *houtenae* (IV)	Ectothermic animals and the environment
S. enterica subsp. *indica* (VI)	Ectothermic animals and the environment
Salmonella bongori (V)	Ectothermic animals and the environment

According to the White-Kauffmann-Le Minor scheme

According to Quinn et al. (1994), *Salmonella* can survive for up to 9 months in the environment (e.g. soil, faecal material, food and water). However, it is unclear whether *Salmonella* found in the environment represent free-living organisms or contaminants from animal faeces since *Acanthamoeba* (free-living amoebae) species seem to play an important role as reservoirs of *Salmonella* in the environment (Bleasdale *et al.* 2009 ; Hadas *et al.* 2004). As of 2007, under the White-Kauffmann-Le Minor scheme (Grimont & Weill 2007), there were 2,579 serovars that belonged to two accepted species: *Salmonella enterica* (2,557 serovars) and *Salmonella bongori* (22 serovars) (Table 4-2). Serovars within the *Salmonella enterica* subsp. *enterica* will be indicated as '*S. enterica* ser. [Serovar name]', while serovars of other subspecies of *Salmonella enterica*, as well as serovars within the *Salmonella bongori* species are indicated by their group (II, IIIa, IIIb, IV, VI and V) and their antigenic formula (e.g. II 56:b:[1,5], IV 6,7:z_{36}:-, V 48:z_{41}:-), as recommended by the World Health Organisation (WHO) Collaborating Center for Reference and Research on *Salmonella* (Grimont & Weill 2007). This taxonomy and the associated methods of reference are illustrated with a flow diagram (Figure 4-1).

Table 4-2 Number of serovars in each species and subspecies according to the White-Kauffmann-Le Minor scheme (Grimont & Weill 2007)

Salmonella species, subspecies (group)	No. of serovars
Salmonella enterica	2,557
S. enterica subsp. *enterica*	1,531
S. enterica subsp. *salamae* (II)	505
S. enterica subsp. *arizonae* (IIIa)	99
S. enterica subsp. *diarizonae* (IIIb)	336
S. enterica subsp. *houtenae* (IV)	73
S. enterica subsp. *indica* (VI)	13
Salmonella bongori (V)	22
Total (genus *Salmonella*)	2,579

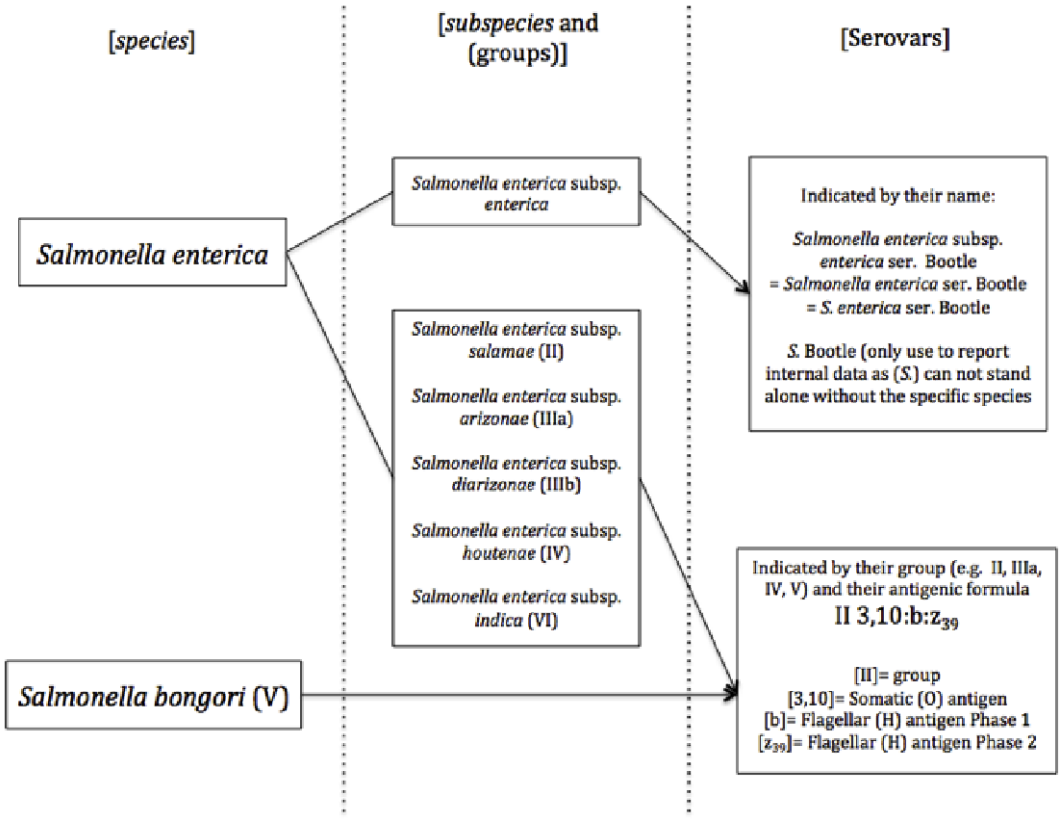

Figure 4-1 Taxonomy and reference methods for *Salmonella* under the White-Kauffmann and Le Minor Scheme. For serovars of *S. bongori* species, the symbol "V" is used to avoid confusion with serovar names of the *S. enterica* subsp. *enterica* group (Grimont & Weill 2007)

The current knowledge of *Salmonella* and its multiple clinical presentations in mammalian veterinary medicine comes, for the most part, from the research that has been undertaken on cattle (Cummings *et al.* 2009 ; Mohler, Izzo, & House 2009 ; Nielsen 2013), pigs (Bergeron *et al.* 2009 ; Oliveira, Carvalho, & Garcia 2006 ; Watson *et al.* 2000), poultry (Chappell *et al.* 2009 ; Duffy, Dykes, & Fegan 2012), horses (Dallap Schaer *et al.* 2012 ; Hartnack, van Metre, & Morley 2012), dogs and cats (Bagcigil *et al.* 2007 ; Weese 2011), and mice (Tam *et al.* 2008). Although conjunctival transmission in experimentally-infected cats (Fox *et al.* 1984) and airborne transmission in pigs (Oliveira, Carvalho, & Garcia 2006) have also been documented, the main route of infection is by ingesting the organism (Quinn *et al.* 2011).

Clinical disease is commonly triggered by stressful events or conditions that could include sudden changes in diet, deprivation of feed, parturition, transportation, heat stress, and drought (Barrow 2012 ; Hirsh 2004 ; Quinn *et al.* 2011). However, the severity of the disease is dependent on the combination of host factors (e.g. age, concurrent disease, immune status and the health of the normal flora of the gastrointestinal tract), and *Salmonella* factors (e.g. strain and infective dose) (Hirsh 2004 ; Radostits *et al.* 2010). Salmonellosis can vary from a subclinical carrier state to an acute endotoxemia and sepsis that is usually fatal (Barrow, Jones, & Thomson 2010 ; Quinn *et al.* 2011). Clinical signs tend to be more severe in young animals than in adults due to an underdeveloped immune system, however, fulminant peracute salmonellosis can still occur in adult animals. The enteric form, unless subclinical or latent, usually presents with fever, depression, and diarrhoea or dysentery, accompanied by dehydration (Hirsh 2004 ; Quinn *et al.* 2011 ; Radostits *et al.* 2010), together with changes in the haemogram [e.g. elevated packed cell volume (PCV) and either neutropenia or neutrophilia] (La Ragione *et al.* 2013) and blood chemistry (e.g. elevated urea nitrogen, and decrease of calcium and protein) (Santos *et al.* 2002). However, the disease can have other presentations, including abortion, meningitis, osteomyelitis and gangrene (Barrow, Jones, & Thomson 2010 ; Markey *et al.* 2013). If endotoxemia is present (systemic form), clinical signs may include high fever, hyperaemic mucous membranes, severe depression, neutropaenia, and leukopaenia (characteristic in horses) (Mackay 2008 ; Radostits *et al.* 2010).

Human salmonellosis has been linked in the past with wildlife and various pathways to infection have been studied, including direct contact, contamination of food by wildlife faeces, consumption of wildlife meat products and indirectly by contact with domestic species following interactions with wildlife species (Hilbert *et al.* 2012). Outbreak reports of salmonellosis in humans by direct contact with wildlife are numerous and wild reptiles and birds are most commonly implicated (Hilbert *et al.* 2012). According to the National Notifiable Diseases Surveillance System (NNDSS) of the Australian Government Department of Health and Ageing (Department of Health and Ageing. Australia 2013), a total of 18,447 cases of salmonellosis have been reported in Western Australia (WA) over the last 22 years (1991 to 2013), with 1,170 cases of human salmonellosis reported in 2012. However, these numbers are an under-representation because not every case would be reported or diagnosed.

Even though the specific sources of contamination are not always identified, macropods and other marsupials may have been the source of infection in at least some of these instances. An example of this is an outbreak of *S. enterica* ser. Paratyphi B, variant Java in

New South Wales, Australia, that was linked to the ingestion of sand that appeared to have been contaminated by long-nosed bandicoots (*Perameles nasuta*). The study tested cloacal swabs and faecal samples from 261 local free-ranging animals of various species, including long-nosed bandicoots (*Perameles nasuta*), black ducks (*Anas superciliosa*), black rats (*Rattus rattus*), and brushtail possums (*Trichosurus vulpecula*) as well as a domestic dog (*Canis familiaris*). Thirty-four isolates were recovered; the majority of these were obtained from the bandicoots, which at the time of sampling did not have any overt signs of disease (Staff *et al.* 2012). Although this study does not provide irrefutable evidence that the long-nosed bandicoot was the source of the outbreak, it does highlight that marsupials should be considered as reservoirs, and therefore potential sources of infection to humans, warranting active surveillance.

Salmonella was first identified in quokkas from Rottnest Island in 1972 (Yadav, Stanley, & Waring), although there was only a low prevalence recorded. They examined the microbial flora of the gut of pouch-young (n= 40, 1-157days old) and pouches of adult females (n= 6, with and without pouch-young), held with *ad libitum* access to food and water. *S. enterica* ser. Newport was isolated from a single 10 day-old pouch-young. The health status of these animals was not assessed and the low prevalence of *Salmonella* should be interpreted cautiously due to the non-specific isolation techniques used, and the *ad libitum* access to food and water. This is so, particularly because of later findings by Hart (1980) and Hart et al. (1985) showing that *Salmonella* appeared to not proliferate when quokkas were given adequate quality and quantity of food and water.

The first case of human salmonellosis [5] linked to *S. brachyurus* in WA was recorded in 1973, in a 14-month old child visiting Rottnest Island; this case was attributed to the consumption of faeces of animal origin and sparked greater interest in the incidence of *Salmonella* on the island (Iveson & Bradshaw 1973). Iveson and Bradshaw (1973) tested (by enrichment and culture methods) rectal swabs and faecal pellets from *S. brachyurus* (n= 87 individuals) as well as cloacal samples from silver gulls (*Larus novaehollandiae*) (n= 83 individuals) from around the site where the child was seen handling faecal pellets as well as other parts of the island. *S. enterica* ser. Javiana was isolated from the child, the rectal swabs of four *S. brachyurus*, but not the silver gulls. Of the quokkas sampled, 71% (62/87) were positive to *Salmonella* spp., with 100 isolates (92 isolates of 17 serovars of *S. enterica* subsp. *enterica* and eight isolates of three serovars of *S. enterica* subsp. *arizonae* [6]; Table 4-3). Mixed infections were present in four faecal samples collected from the

[5] defined as the disease that is associated with the abnormal proliferation and tissue invasion of *Salmonella* (Barrow, Jones, & Thomson 2010).
[6] at the time this work was published, the term "*Arizona*" referred to a unique genus of bacteria. Since then, it has become accepted that Arizona is *S. enterica* subsp. *arizonae* (Brenner *et al.* 2000).

ground (random locations), but not in cloacal swabs. With the use of enrichment and culture media specific for *Salmonella*, this study revealed high infection rates with multiple serovars of *Salmonella* in quokkas on Rottnest Island, and drew attention to the potential significance of the species as a natural carrier and source of *Salmonella*.

Subsequently, a 10-year surveillance program was started. This initiative revealed important information about the ecology of *Salmonella* on Rottnest Island (Hart, Bradshaw, & Iveson 1985, 1986 ; Hart, Iveson, & Bradshaw 1987 ; Iveson, Bradshaw, & Hart 2007 ; Iveson & Hart 1983). Samples were collected from *S. brachyurus*, reptiles, birds, domestic animals (horses and donkeys) and water sources. To date, a total of 55 [7] different *Salmonella* serovars have been isolated from *S. brachyurus* (Table 4-3). Iveson and Hart (1983), using enrichment and culturing methods, identified *Salmonella* in 1,551 out of 4,038 *S. brachyurus* that were tested (i.e. 38% prevalence). A total of 40 unique serovars were identified, of which the most prevalent serovars were *S. enterica* ser. Muenchen (recovered from 484 individuals; 33.6%) and *S. enterica* ser. Newington (recovered from 291 individuals; 18.7%) (Table 4-3). The prevalence of *Salmonella* was lower for birds (10/417; 2.4%), but higher for reptiles (50/76; 66%) and domestic animals (40/45; 88%) tested. The serovars recovered from birds, reptiles and domestic animals were similar to those isolated from *S. brachyurus*, indicating that *Salmonella* is both abundant on Rottnest Island and widespread among different animal taxa and ecosystems.

Hart (1980) determined that *Salmonella* infections in *S. brachyurus* had a seasonal variation, with low shedding rates (0-30%) in winter and high shedding rates (70-100%) during summer. In contrast, infection rates in areas where quokkas had access to supplementary food sources (e.g. near towns) were maintained throughout the year at 0-30%. The author suggested that this seasonal variation in infection rate may be explained by the 'urban' *S. brachyurus* being able to avoid nutritional stress during the driest part of the year. The results of a subsequent experiment (Hart, Bradshaw, & Iveson 1985) supported this suggestion. Two groups of *S. brachyurus* (group sizes were not published) were kept in natural habitats on Rottnest Island for over a year. One group (experimental) was supplemented with *ad libitum* food and water while the second (control) group was not and therefore was subject to the natural seasonal shortages. The food-supplemented group had one third of the *Salmonella* proliferation compared with the control group. Proliferation of *Salmonella* increased almost immediately after food and water supplementation was stopped for the experimental group to become similar between the two groups by the third week after withdrawal of food and water. The authors ascribe the

[7] includes only serovars with an updated antigenic formulae, and serovars isolated and serotyped in this study

proliferation of *Salmonella* to an increase in gastric pH, which is thought to be due to a decrease in the production of short-chain fatty acids that occurs when the animal faces a reduction in food quality and availability. It is not known how *Salmonella* proliferation and shedding is linked to gastric pH in *S. brachyurus*, but in ruminants (also foregut fermenters), a low pH in the rumen is unfavourable to *Salmonella* growth (Radostits *et al.* 2010). Faecal excretion in *S. brachyurus* was typically 10^5 CFU/g faeces during summer and never exceeded 10^6 CFU/g faeces (Hart, Bradshaw, & Iveson 1985). For comparison, excretion rates of 10^5 CFU/g faeces are common in cattle with subclinical salmonellosis (Barrow, Jones, & Thomson 2010). In production animals, stress factors (e.g. transition diets and heat stress) likely contribute to the proliferation of *Salmonella* in the gastrointestinal tract, and therefore the increase in faecal excretion rates. Morphometric data for quokkas [i.e. Body Condition Index (BCI)] also showed a seasonal pattern coinciding with the proliferation of *Salmonella*, with a poor BCI in summer (high incidence of *Salmonella*) attributed to the progressive starvation, compared with winter (low incidence of *Salmonella*) (Hart, Bradshaw, & Iveson 1985). However, no direct correlation between *Salmonella* proliferation and BCI was found, which may have been because *Salmonella* excretion rates increased before the BCI decreased.

According to Hart (1980), on Rottnest Island, proliferation of *Salmonella* seemed to occur more rapidly in females than in males, however, most animals will ultimately get infected. Iveson et al. (2007) obtained similar results where females captured at a particular location on the island had a significantly higher infection rate than males. However, at a greater scale, there was no significant difference between the proportion of infected females and males on Rottnest Island. In contrast to these findings, it is believed that males are more susceptible than females to infectious diseases due to the immunomodulatory effects of sex-steroid hormones (Billingham 1986 ; Chrousos 2010 ; Zuk & McKean 1996). For example, the study by Tomlinson et al. (2013) in badgers infected with *M. bovis*, determined that females were more resilient to active infection than males, therefore living longer after bacterial excretion was detected, while a shorter survival rate was observed in males. This shorter survival rate of *M. bovis*-infected males was suggested to be associated with testosterone-related immunosuppression. In mice infected with *Giardia muris*, Daniels and Belosevic (1994) found that females had a stronger IgG2b and IgG3 response than males, and stopped shedding cysts 20 days post infection, males on the contrary shed cysts for over 60 days. However, susceptibility in females to infectious diseases can also increase under certain events such as during pregnancy and lactation. For instance, Festa-Bianchet (1989) found that lactating big horn (*Ovis canadiensis*) ewes had greater faecal counts of lungworm larvae than non-lactating

females; additionally, females that had given birth by two years of age were more likely to die during an outbreak of pneumonia when compared to females that had not lambed by the same age. Although testosterone is generally associated with decreased immunity and oestrogen with increased immunity, McClelland and Smith (2011) showed, that the effects of sex-steroids on the immune system are closely related to the type of organism involved. In the context of *Salmonella,* the severity of the infection is a multifactorial event and includes factors such as the immune status of the animal, the initial load of *Salmonella*, and the strain or strains involved (Quinn *et al.* 2011).

Table 4-3 *Salmonella* [1] serovars isolated from *Setonix brachyurus* from faecal pellets (F) (collected from the animal A, post-mortem PM, ground G, or undetermined U), Rectal swabs (R), Intestinal (I), and Undetermined (U) samples from Rottnest Island (RI), Bald Island (BI) or the mainland (ML).

Serovar		Yadav et al. (1972) [2]	Iveson and Bradshaw (1973)	Hart et al. (1982)	Iveson and Hart (1983)	Hart et al. (1985)	Hart et al. (1986)	Iveson et al. (2007) [3]
Nomenclature								
Original paper	White-Kauffmann-Le Minor	RI	RI	RI	RI	RI	ML & BI	RI
S. adelaide	*S. enterica* ser. Adelaide		R	R F^{U}	U	G F^{PM}		R
S. alsterdorf or *II alsterdorf* [4]	II 1,40:g:[m],[s],t:[1,5]			R F^{U}	U			R
	S. enterica ser. Alachua							
S. anatum	*S. enterica* ser. Anatum		G		U			R
S. bahrenfeld	*S. enterica* ser. Bahrenfeld		R	R F^{U}	U			R
S. birkenhead	*S. enterica* ser. Birkenhead							R
S. bleadon	II 17:g,t:[e,n,x,z$_{15}$]			R F^{U}	U			
S. blukwa	*S. enterica* ser. Blukwa			R F^{U}	U			
S. bootle	*S. enterica* ser. Bootle		R		U			R
S. bovis-morbificans	*S. enterica* ser. Bovismorbificans			R F^{U}	U		R (BI)	R
	S. enterica ser. Bredeney							
S. bunnik	II 43:z$_{42}$:1,5,7			R F^{U}	U			
S. carnac	*S. enterica* ser. Carnac						R G (BI)	
S. charity	*S. enterica* ser. Charity						R G (BI)	
	S. enterica ser. Cerro							
S. chester	*S. enterica* ser. Chester		R G	R F^{U}	U			R
S. decatur	*S. enterica* ser. Choleraesuis var. Decatur		G	R F^{U}	U			R
S. derby	*S. enterica* ser. Derby			R F^{U}	U			
S. fremantle	II 42:g,t:-		R		U			
S. give	*S. enterica* ser. Give		R	R F^{U}	U			
S. havana	*S. enterica* ser. Havana		R G	R F^{U}				
S. infantis	*S. enterica* ser. Infantis			R F^{U}	U			R
S. javiana	*S. enterica* ser. Javiana		R G	R F^{U}	U	F^{PM}		R
S. merseyside	II 16:g,t:[1,5]						G (BI)	
S. muenchen	*S. enterica* ser. Muenchen		R G	R F^{U}	U	R F^{PM}		R
S. newbrunswick	*S. enterica* ser. Give var. 15^{+}		R				G (ML)	
S. newington	*S. enterica* ser. Newington		R G	R F^{U}		R F^{PM}		R
S. newport	*S. enterica* ser. Newport	I^{PM}		R F^{U}	U			
S. oranienburg	*S. enterica* ser. Oranienburg		R	R F^{U}	U	R		R
S. orientalis	*S. enterica* ser. Orientalis		R	R F^{U}	U	F^{PM}	G (ML)	R
S. orion	*S. enterica* ser. Orion		R	R F^{U}	U			R

Serovar		Yadav et al. (1972) [2]	Iveson and Bradshaw (1973)	Hart et al. (1982)	Iveson and Hart (1983)	Hart et al. (1985)	Hart et al. (1986)	Iveson et al. (2007)[3]
Nomenclature								
Original paper	White-Kauffmann-Le Minor	RI	RI	RI	RI	RI	ML & BI	RI
S. potsdam	*S. enterica* ser. Potsdam			R F^{U}	U			R
S. rottnest	*S. enterica* ser. Rottnest			R F^{U}	U			R
S. saint-paul or saintpaul	*S. enterica* ser. Saintpaul			R F^{U}	U			
S. singapore	*S. enterica* ser. Singapore			R F^{U}	U	F^{PM}		
S. typhimurium	*S. enterica* ser. Typhimurium		R G	R F^{U}	U	R	G(BI)	R
S. wandsbek or *II wandsbek*[5]	II 21:z_{10}:z_6		R	R F^{U}	U	R F^{PM}		R
S. waycross or *IV waycross*[6]	*S. enterica* ser. Waycross		R	R F^{U}	U	R F^{PM}		R
S. 6,8:-:- (O Group C_2)	-						R (BI)	
S. 48:d:-	-			R F^{U}	U			R
53:d:z_{42}	II 53:d:z_{42}							R
Arizona spp.					U			
IIIb 25:l v:z_{53}	-							R
IIIb 50:K:z_{35}	IIIb 50:K:z_{35} [7]							R
IIIb 61:l v:z_{35}	IIIb 61:l,v:z_{35}							R
IIIb 61:z_{52}:z_{53}	IIIb 61:z_{52}:z_{53}							R
A. 9:26:21	IIIb 50:z_{52}:z_{35} [7]				U			
A. 9:26:31	-				U			
A. 9:29:21	-				U			
A. 9a9b:26-21	IIIb 50:z_{52}:z_{35} [7]			R F^{U}				
A. 9a9b:26-31	IIIb 50:z_{52}:z [8]			R F^{U}				
A. 9a9b:29-21	IIIb 50:K:z_{35} [7]			R F^{U}				
A. 16:23:25	IIIb 38:lv:z53 [9]		R		U			
A. 16:26:25	IIIb 38:z52:z53 [9]				U			
A. 20:22-21	IIIb 35:(k):z35 [9]			R F^{U}				
A. 20:29:21	-				U			
A. 20:29:25	IIIb 35:(k):z_{53}:[z_{50}] [10]			R F^{U}	U			
A. 26:23:21	IIIb 61:l,v:z_{35} [7]		R	R F^{U}	U			
A. 26:23:25	-		R					
A. 26:26-25	IIIb 61:z52:z53 [9]			R F^{U}				
A. 28:32:28	IIIb 47:c:e,n,x,z15 [8]			R F^{U}	U			

[1] Serovars previously classified as genus *Arizona* (A.) are included in this table because they have since been reclassified into the genus *Salmonella*

[2] *Salmonella* was isolated from a 10-day old pouch young at post-mortem

[3] In this study, samples were only collected from *S. brachyurus* that had been captured at sites considered to have high human-animal interaction rates i.e. settlements, camping areas etc,

[4] This serovar was referred to as *S. alsterdorf* in Iveson and Bradshaw (Iveson & Bradshaw 1973) and *II alsterdorf* in Iveson et al. (Iveson, Bradshaw, & Hart 2007)

[5] This serovar was referred to as *S. wandsbek* in Iveson and Bradshaw (1973) and *II Wandsbek* in Iveson et al. (2007)
[6] This serovar was referred to as *S. waycross* in Iveson and Bradshaw (1973) and *IV Waycross* in Iveson et al. (2007)
[7] Current *Salmonella* antigenic formula was obtained from Hall and Rowe (1992)
[8] Patricia Fields, CDC Atlanta *pers. comm*. 2013
[9] Damien Bradford, PathWest, Perth *pers. comm.* 2013
[10] Current *Salmonella* antigenic formula was obtained from Weis et al. (1986)

In Australian macropods, salmonellosis typically presents as sepsis or gastroenteritis, and is most commonly noted in captive animals (Vogelnest & Portas 2010). However, evidence suggests that macropods are asymptomatic carriers of *Salmonella* (Hart, Bradshaw, & Iveson 1985 ; Potter, Reid, & Fenwick 2011 ; Speare 1988a). Speare (1988a) recovered *Salmonella* in faeces from 37/138 (26.8%) orphaned joeys of several species of the genus *Macropus* and *Wallabia*, where it was only sometimes associated with diarrhoea (faeces containing traces of blood). This suggests that the remainder of joeys with no clinical signs were therefore asymptomatic carriers of *Salmonella*. *Salmonella* has also been recovered from free-ranging macropods destined for the meat industry. Potter et al. (2011) studied the prevalence of naturally acquired *Salmonella* in commercially harvested (for human consumption) wild-caught western grey kangaroos (*Macropus fuliginosus*). *Salmonella* (II 42:g,t and various serovars of *S. enterica* subsp. *enterica*: Muenchen, Kiambu, Rubislaw, Lindern, Champaing and Saintpaul) was cultured in 23/645 (3.6%) of animals. Shedding was significantly higher during April – June during which time, faecal samples were not properly formed, were green and contained large numbers of intestinal worms. However, there appeared to be little correlation between the presence of *Salmonella* and the colour and consistency of the stools. Although the general health of these animals was difficult to assess (the kangaroos were shot and eviscerated in the field by professional hunters), it seems likely that at least some of these kangaroos may have been asymptomatically carrying *Salmonella*.

With this evidence, it seems reasonable to infer that macropods could carry a variety of *Salmonella* spp. in their digestive tract, many of which are likely zoonotic pathogens, without obvious clinical signs of disease. Iveson et al. (2007) suggested that quokkas are carriers of *Salmonella* since recaptured animals remained positive to the same serovar for up to nine months, while Hart et al. (1985) and Iveson et al. (1983) reported that "the vast majority of infections are believed to be non-disease state" even though no animal was physically examined. However, the absence of external clinical signs of disease is insufficient evidence to support the absence of disease. For this reason, we aimed to correlate *Salmonella* infection with the presence of disease in the Rottnest Island quokka. The presence of disease was investigated through, physical examination, HMT and BLC profiles. We also included confounding factors such as the presence of concomitant infection with other organisms. Additionally, we extended our study to mainland WA, by including animals from selected locations.

4.3 Materials and Methods

Between September 2010 and December 2011, 153 quokkas were captured, of which 92 were tested for *Salmonella* (71 on Rottnest Island and 21 on the mainland). For trapping sites descriptions, general procedures and blood sampling techniques, refer to Chapter 3, section 3.3. Animals were examined and tested for *Salmonella* in faecal samples by culture, and isolates were finally characterised by biochemical methods and serotyping.

Faecal samples [8] for the isolation of *Salmonella* were obtained by rectal palpation using examination gloves. The external area around the cloaca was cleaned with a 1:1 mixture of chlorhexidine gluconate and 70% ethanol before the procedure. Some animals spontaneously defecated after rectal palpation, however, even though the pellet was not collected directly from the rectum, the sample was still included in this study. In no cases were faecal samples taken from cotton/hessian bags, or traps. Faecal samples were placed into 5 mL polycarbonate yellow cap sterile tubes (SARSTEDT Aktiengeseilschaft & Co. Germany) and stored at 4 ˚C until processing. Nematode eggs and *Eimeria* sp. oocysts were screened by light microscopy (see Chapter 3, section 3.4.1).

Blood samples from the lateral tail vein were collected into EDTA and lithium heparin blood collection tubes and sent to the Clinical Pathology service of the Murdoch University Veterinary Hospital for HMT and BLC analyses. Differential counts were done manually on blood smears. For specific details on methodologies, please see Chapter 3, section 3.4.2. Several parameters were recorded and used in this study: red blood cell concentration (RBC), haemoglobin concentration (HGB), packed cell volume (PCV), total white blood cell counts (corrected to exclude nucleated red blood cells) (WBC), the absolute concentrations for neutrophils (NEUT), eosinophils (EOS), basophils (BASO), lymphocytes (LYMPH) and monocytes (MONO), and the BLC analytes: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), total protein (PROT), albumin (ALB), calcium (CA), phosphorus (P), cholesterol (CHOL), total bilirubin (BILT), glucose (GLUC), creatinine (CREAT), urea (UREA), and vitamin E (Vit. E). PBCM (red and white blood cells) were visually recorded from blood smears (e.g. keratocytes, schistocytes, reactive lymphocytes, and atypical neutrophils -including toxic changes-). Microfilariae were screened for from blood smears, while *Theileria* sp. and *Babesia* sp. were screened for nPCR (see Chapter 7, section 7.3.1). Macropod herpesvirus 6 (MaHV-6) was screened for by nPCR (see Chapter 6, section 6.3.1), and *Cryptococcus* spp.

[8] faecal pellets were used instead of rectal swabs as this type of sample produced better results when doing isolations from samples with a low number of *Salmonella* (Hart, Bradshaw, & Iveson 1985)

were screened by culture methods and isolates characterised by PCR (see Chapter 5, section 5.3.1).

4.3.1 *Laboratory techniques for Salmonella*

4.3.1.1 Isolation

In line with WHO recommendations, manipulation of samples and all diagnostic procedures were done in a class II laminar flow biosafety cabinet (LAF Technologies Pty Ltd., North Ringwood, Australia) (Chosewood & Wilson 2009). Initial screening of faecal samples for the presence of *Salmonella*, was achieved using a direct method with pre-enrichment by inoculating 0.5 g of faecal matter into buffered peptone water (PW) (pre-enrichment) which were then incubated at 37 ˚C for 20 h. Fresh Difco™ xylose lysine deoxycholate (XLD) agar plates (BD Diagnostics, Maryland, USA) were then lawn inoculated and incubated at 37 ˚C for at least 24 h. Buffered peptone water was used to resuscitate *Salmonella* that had been damaged by conditions such as prolonged storage and freeze-thawing (Davies 2013). XLD is a selective and differential medium used in the isolation and differentiation of enteric pathogens from clinical specimens (Markey *et al.* 2013 ; Nataro *et al.* 2011). It contains xylose, which is fermented by practically all Enterobacteriaceae except *Shigella* species, as well as lysine that enables *Salmonella* to be differentiated from the non-pathogenic Enterobacteriaceae by slowing down the fermentation process of *Salmonella* on the xylose (Zimbro *et al.* 2009). The agar also contains an indicator system allowing the visualisation of hydrogen sulphide (H_2S) produced by the majority of *Salmonella* serovars, resulting in the formation of a black precipitate, and inhibits gram-positive organisms by the presence of sodium desoxycholate (Zimbro *et al.* 2009).

An indirect method with delayed secondary enrichment (DSE) was used for the isolation and identification of the isolates, as well as for the subjective assessment of the bacterial load. This was achieved by inoculating vials of Rappaport Vassiliadis (RV) enrichment broth (Oxoid LTD., Hampshire, England) with 10 µL of PW-faecal mixture (from the direct method) that were then incubated at 42± 1 ˚C for 48 h in a water bath. Rappaport Vassiliadis contains malachite green and magnesium chloride that inhibits other organisms and is selective for *Salmonella* (Rappaport, Konforti, & Navon 1956 ; Vassiliadis *et al.* 1978 ; Zimbro *et al.* 2009). Fresh XLD plates were then inoculated with 30 µL of the PW-RV enrichment broth suspension, streaked out for single colonies and incubated for 18-24 h at 37 ˚C. The use of a pre-enrichment step together with a selective enrichment broth for *Salmonella* has been recommended for the detection of the bacterium in

subclinical animals (Davies 2013). Colonies with typical morphology appear mostly red (although pink is possible) with or without pale/dark black centres (Wallace, Jacobson, & Hammack 2009), although the majority of *Salmonella* serovars typically produced a black precipitate (Markey *et al.* 2013). If typical colonies were not present, atypical *Salmonella* colonies (i.e. light yellow or yellow colonies with or without black centres) were then examined, if present (Wallace, Jacobson, & Hammack 2009). To allow for the determination of multiple infection, multiple suspicious colonies were harvested and subcultured from each inoculated XLD plate. Other morphological characteristics of *Salmonella* colonies (e.g. surface, structure, edge, light properties, and opacity) were considered but not as a definitive selection criterion. Growth on XLD plates was scored as confluent (no separate colonies), heavy (>200 colonies), moderate (50-200 colonies), and light (<50 colonies or number of colonies). Suspicious colonies were then subcultured onto Nutrient Agar (NA) plates and incubated for 24 h at 37 ˚C for further processing. Detailed protocols for the direct method with pre-enrichment, indirect method with DSE and the preparation of the XLD agar plates, are provided in Appendix 10.1.1.

4.3.1.2 Preliminary identification and storage

Suspicious colonies subcultured in NA were first confirmed as Gram negative bacilli by Gram's staining. These isolates were subsequently characterised by a set of biochemical tests [indole (I), methyl red (MR), Voges-Proskauer (VP), citrate (C), urea hydrolysis (U), lactose fermentation (L) and lysine decarboxylase (LD)] to allow discrimination between *Salmonella* and two other bacteria with similar morphology in XLD, *Shigella* sp. and *Proteus* sp. Figure 4-2 illustrates the step by step protocol while Table 4-4 shows the different biochemical signatures between *Salmonella* sp., *Shigella* sp. and *Proteus* sp. Lastly, isolates were confirmed as *Salmonella* by an antiserum agglutination test using Antiserum Salmonella Omnivalent Omni-O (A-60) and Antiserum Salmonella Polyvalent OMG (both from Bio-Rad laboratories, Marnes-la-Coquette, France). These two antisera were used to detect agglutination to the presence of somatic (O) antigen of groups O:2 to O:60, and groups O:60 to O:67, respectively. Isolates were subsequently stored for further analysis in Protect® Bacterial Preservers (Technical Service Consultants Limited, Lancashire, United Kingdom) cryovials according to the manufacturer's guidelines. For specific details on these protocols, refer to Appendix 10.1.2.

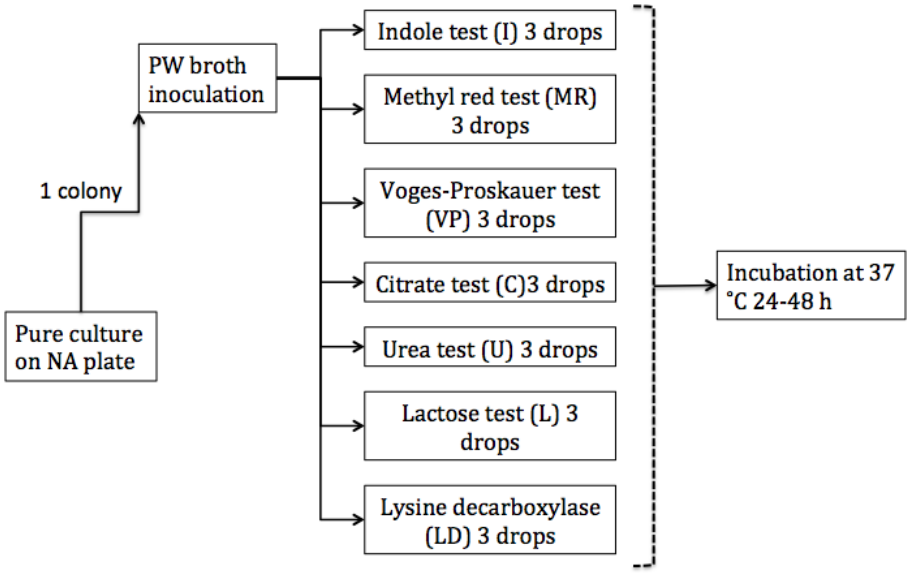

Figure 4-2 Biochemical profiling tests used to do a preliminary identification of the suspicious *Salmonella* colonies.

Table 4-4 Comparative biochemical profiling used to differentiate *Salmonella*, *Proteus*, and *Shigella* isolates. Adapted from Markey et al. (2013)

Bacteria	I	MR	VP	C	U	L	LD
Salmonella	-	+	-	+	-	-	+
Proteus mirabilis	-	(+)	-	d	+	-	-
Proteus vulgaris	+	+	-	(-)	+	-	-
Shigella sp.	V	+	-	-	-	-	-
Expected result for *Salmonella*	Yellow	Red	Colourless/Yellow	Blue	Yellow	Red/Pink	Purple + turbid

+ = 90-100% strains positive, (+) = 76-89% positive, d = 26-75% positive, (-) =11-25% positive, - = 0-10% positive, v =reaction varies between species

4.3.1.3 Revival and serotyping

Isolates frozen at -80 ˚C, were revived by incubation in nutrient broth (NB) at 37 ˚C for 24 h (see Appendix 10.1.3 for details). Upon confirmation of growth by turbidity in the medium, NA and XLD agar plates were inoculated and streaked out for single colonies, and incubated at 37 ˚C for 24 – 48 h. For quality control purposes, morphology on XLD was cross matched with previous records, and a colony on NA plate was then subcultured onto an NA slant, and incubated at 37˚C for 18 – 24 h. Isolates were then submitted to the national reference laboratory for *Salmonella* in Perth at PathWest, Sir Charles Gairdner Hospital, WA for serotyping by antisera slide agglutination (Kauffmann-White-LeMinor scheme) to detect O (somatic), H (flagellar) and K (capsular) antigens. Two isolates were sent to the Australian Salmonella Reference Centre (Institute of Medical and Veterinary Science; IMVS Pathology) for further serotyping.

4.3.2 *Statistical analyses*

Haematology, BLC, and PBCM datasets for Rottnest Island animals that tested positive or negative to *Salmonella*, were analysed independently due to differences in sample sizes between these datasets (some individuals were tested for either haematology or blood chemistry, and not all individuals had a blood smear, which would mean their exclusion from analyses of the entire dataset due to missing data). *Season* and *sex* were used as covariates in all three sets of analyses. Mainland data was not included as only one animal tested positive to *Salmonella*, making the effective sample size difference between mainland and Rottnest Island too unbalanced. Data were explored visually with non-metric Multi-Dimensional Scaling (nMDS) and a Bray-Curtis similarity measure (Bray & Curtis 1957) using PAST v. 3.02 (Hammer, Harper, & Ryan 2001). Haematology and blood chemistry response variables were fitted to an approximate normal distribution (BoxCox transformation; STATISTICA v. 9.1, StatSoft Inc.).

Haematology and BLC dependant variables were range-standardised to a scale between 0 and 1, while PBCM were not as it was a binary dataset. For each nMDS plot, two-or-three-dimensional analyses were selected according to the model that had the lowest stress statistic to determine adequacy of the fit. To determine the similarity or dissimilarity of the HMT, BLC and PBCM datasets between the groups of interest (*Salmonella*-positive and *Salmonella*-negative), a pairwise similarity percentage (SIMPER, PAST v. 3.02) analysis (Clarke 1993), using Bray-Curtis similarity measure (Bray & Curtis 1957) was carried out. SIMPER results (i.e. percent of contribution of each variable to the similarity or dissimilarity) are accompanied by the arithmetic mean ($\bar{x}$) and standard deviation (SD) for each HMT and BLC variable, while odds ratio (OR) and 95% confidence intervals (CI) were calculated (Woolf 1955) for each PBCM observed in blood smears. To determine the significance of any differences between dependant variable communities (HMT, BLC and PBCM) as a function of *Salmonella*, a two-way non-parametric permutational multivariate analysis of variance (Two-way PERMANOVA, PAST v. 3.02) (Anderson 2001) with 9,999 permutations was subsequently run. This test provides a *Pseudo-F* statistic that is used to compare variability between groups from variability within groups, in which the larger the value of *F*, the more likely is that the null hypothesis of no variability between groups is false (Anderson 2001). A *p*-value is then used to detect the significance of each factor in the design and the interaction between them. The effect size (ES) $d_{Cohen's}$ was calculated for $p = 0.05$, or for values that were 0.01-0.02 points above the cut off (< 0.05), either by using the *F*-value given by PERMANOVA or the χ^2 value given by Chi-square (Lipsey & Wilson 2001 pp. 172-188). Clinically, the magnitude of the ES was considered to be small if $d \leq 0.2$, moderate if $0.2 > d < 0.8$ and large if $d \geq 0.8$ (Cohen 1988 pp. 531-537).

Seasonal differences in the prevalence of *Salmonella* infections on Rottnest Island were explored using χ^2 for Trend. Associations between *Salmonella* and *sex* (across Rottnest Island) as well as associations between *Salmonella* and concomitant infection with *Eimeria* sp., macropod herpesvirus 6 (MaHV-6), *Theileria* sp., *Babesia* sp., *Cryptococcus* spp., microfilariae, trypanosomes and nematode eggs, were explored using χ^2 with Yates' correction. Odd ratios and 95% CI were calculated using the Woolf's method (1955). When a null value was present in a contingency table, 0.5 were added to each observed value in order to calculate OR and 95% CI (Altman 1999). Additionally, ES ($d_{Cohen's}$) was calculated when considered appropriate. Using Fisher's exact test (smaller sample sizes), we also looked at the individual relation between *sex* and *Salmonella* prevalence at each trapping location on Rottnest Island, and at the prevalence of *Salmonella* by the degree of disturbance of their trapping sites, classified as disturbed (constant human interaction, infrastructure is common, unconventional food sources available) and less disturbed (less human interaction, infrastructure still present but less, less or no access to unconventional food sources). All other CI for estimates of proportions (i.e. prevalence), were calculated using the Wilson model for n≤ 40, and the Jeffreys model for n≥ 40 (Brown, Cai, & DasGupta 2001). A T test with Welch's correction was used to explore the differences in WBC between microfilariae-positive and -negative animals within the *Salmonella* sampled group, as well as between *Salmonella*-positive and *Salmonella*-negative individuals. Significance was set at $p< 0.05$ for all analyses, unless stated otherwise.

4.4 Results

4.4.1 *Microbiology notes*

The morphology of all *Salmonella* serovars isolated (at 37 ˚C and 24 h post incubation) in this study was considered to be typical for XLD agar: pink to red with black centres (Wallace, Jacobson, & Hammack 2009) (Figure 4-3). Colonies were mostly circular, effuse, smooth and translucent. They were Gram negative bacilli on Gram's stain, and presented a typical biochemical profile for *Salmonella* (see Table 4-4). All isolates exhibited positive agglutination (Figure 4-3) to antiserum Salmonella Omnivalent Omni-O (A-60) and no agglutination to antiserum Salmonella Polyvalent OMG, indicating isolates were not serovars of the somatic (O) antigen groups 61 to 67.

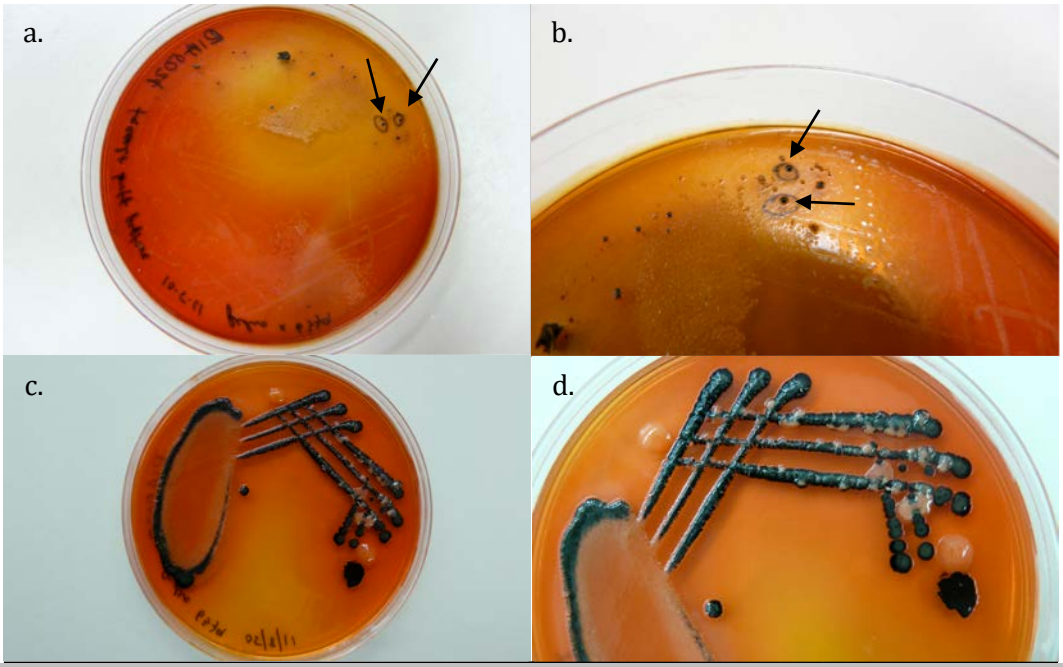

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Figure 4-3 Growth on XLD agar plates and antiserum agglutination test. (a, b) suspicious *Salmonella* colonies on an XLD agar plate inoculated with a suspension of PW (pre-enrichment) and faeces. Colonies are indicated by closed arrows. (c, d) suspicious *Salmonella* colonies on an XLD agar plate inoculated with a suspension of pre-enrichment and Rappaport Vassiliadis selective enrichment broth, and streaked out for single colonies. Note colonies of other intestinal bacteria and the predominant and suspicious colonies with H_2S precipitate. (e) negative agglutination test for *Salmonella*, (f) positive agglutination test for *Salmonella* with obvious clumps in the homogenate.

Of the 35 animals that were positive to *Salmonella* (Rottnest Island= 34, mainland= 1), 80% (28/35, 95% CI 64-90) had a confluent growth on XLD (RV selective enrichment inoculum) at 24 h post incubation, while there was a heavy growth of the isolates from the remaining seven animals (i.e. >200 colonies).

4.4.2 *Distribution and prevalence of Salmonella serovars (Rottnest Island and mainland)*

Of the 21 individuals (18 male and 3 female) trapped from mainland sites that were tested for *Salmonella*, an adult male from Jarrahdale (prevalence 4.8%, CI 0.8-22.7) was positive to *Salmonella* (*S. enterica* subsp. *diarizonae* (IIIb) ser. 50:k:z_{35}). By contrast, of the 71

individuals on Rottnest Island tested (40 male and 31 female), 34 animals were positive to *Salmonella* (prevalence 47.9%, CI 36.5-59.4). Of these 34 animals, 18 were males (prevalence $^{n=40}$ =45%, CI 36.7-68.5) and 16 were females (prevalence $^{n=31}$ =51%, CI 31.5-63.3). The probability (i.e. OR) of females to be positive to *Salmonella* was 1.30 (CI 0.51-3.34) times higher than males. However, this association was not significant ($\chi^2_{1, n=71}$= 0.30, p= 0.581).

There were no significant differences at in the prevalence of *Salmonella* by sex at each individual trapping location within Rottnest Island (Table 4-5), and no significant difference in the prevalence of *Salmonella* in quokkas by the degree of disturbance of their habitat (Fisher's exact test p= 0.456). By contrast, there was seasonal variation ($\chi^2_{1, n=71}$= 7.65, p= 0.001) in the prevalence of *Salmonella* in Rottnest Island animals (Figure 4-4) with the highest prevalence of infection at the end of the dry summer season (March), while the lowest prevalence was at the end of the wet winter (September).

Table 4-5 Two-tailed Fisher's exact test results for sex and presence of *Salmonella* at individual locations where animals were trapped on Rottnest Island, CI= confidence interval.

Trapping sites	+ve ♂/n (prevalence)	95% CI	+ve ♀/n (prevalence)	95% CI	Fisher's exact test
Kingston	3/8 (37.5%)	13.7-69.4	3/4 (75%)	30.1-95.4	p= 0.626
Parker Point	1/6 (16.6%)	3.0-56.4	3/5 (60%)	23.1-88.2	p= 0.569
Serpentine	4/7 (57.1%)	25-84.2	4/5 (80%)	37.6-96.4	p= 0.999
Barker Swamp	4/6 (66.6%)	30-90.3	3/5 (60%)	23.1-88.2	p= 0.999
Settlement	3/7 (42.8%)	15.8-75	1/6 (16.6%)	3.0-56.4	p= 0.603
West End	3/6 (50%)	18.8-81.2	2/6 (33.3%)	9.7-70	p= 0.999

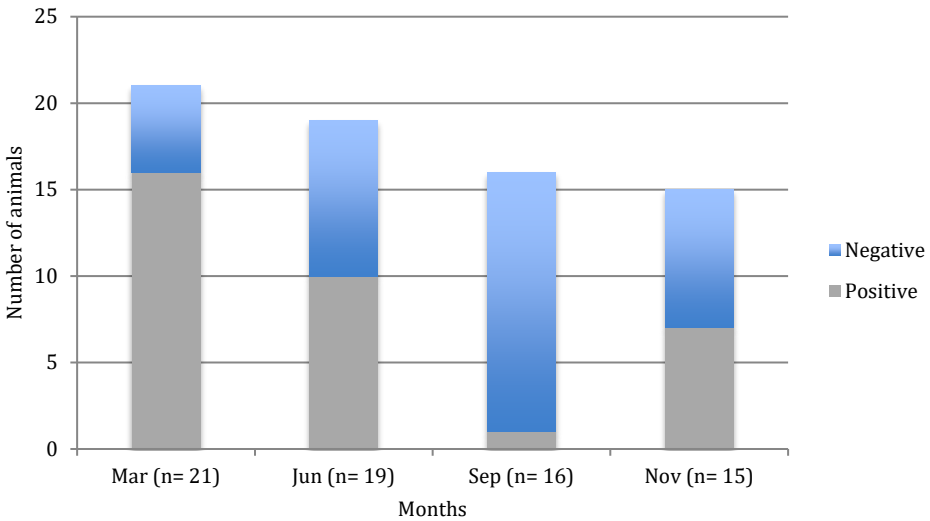


Figure 4-4 Monthly proportion of *Salmonella* spp. positive and negative animals on Rottnest Island between March and December of 2011. N values represent the total number of animals sampled in each month.

A total of 16 serovars were identified in 37 isolations obtained from 35 animals (Rottnest Island and mainland combined). Of these, 13 serovars (81.25%, CI 57-93.4) belonged to *S. enterica* subsp. *enterica*, one serovar (6.25%, CI 1.1-28) to *S. enterica* subsp. *salamae* (II), and two serovars (12.5%, CI 3.2-36) to *S. enterica* subsp. *diarizonae* (IIIb). Of these three subspecies, only *S. enterica* subsp. *diarizonae* (IIIb) was isolated from the mainland (Figure 4-5). Two serovars were recovered simultaneously from two animals from Rottnest Island: *S. enterica* ser. Adelaide and *S. enterica* ser. Cerro were recovered from a male captured at Parker Point, and *S. enterica* ser. Chester with *S. enterica* ser. Cerro from another male captured at Serpentine. The highest prevalences of *Salmonella* in quokkas on Rottnest Island were found at Serpentine (prevalence $^{n=12}$ =66.7%, CI 39.1-86.2) and Barker Swamp (prevalence $^{n=11}$ =63.6%, CI 35.4-84.8), while the lowest prevalence was found at the Settlement (prevalence $^{n=13}$ =30.8%, CI 12.7-57.6). Of 36 isolations obtained on Rottnest Island, the three most frequent serovars were *S. enterica* ser. Adelaide (19.4%, CI 10-35), *S. enterica* ser. Muenchen (13.9%, CI 6.1-29), and *S. enterica* ser. Cerro (11.1%, CI 3.9-25). A total of five new *Salmonella* serovars were isolated from quokkas across Rottnest Island and mainland samples (Table 4-6).

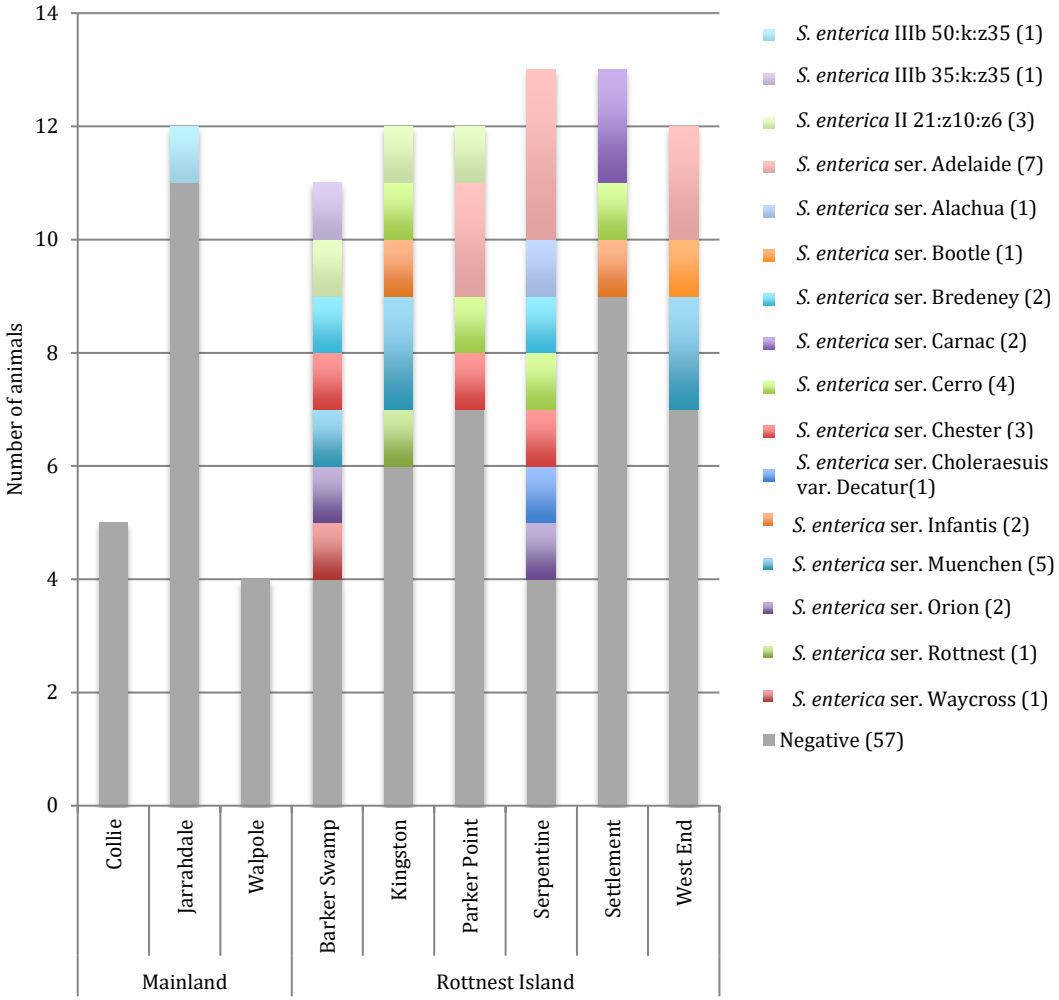


Figure 4-5 Animals tested positive and negative to three subspecies of *Salmonella* [*enterica* (serovar name), *diarizonae* (IIIb), and *salamae* (II)] on six sites at Rottnest Island and four sites on the mainland. The total number of each serovar is indicated in parenthesis.

Table 4-6 Distribution and number of *Salmonella* isolations from quokkas on Rottnest Island (six sites) and the mainland (one site) between June 2010 and December 2011.

Serovar isolated	Barker Swamp	Kingston	Parker Point	Serpentine	Settlement	West End	Mainland	Totals
S. enterica subsp. *enterica*								
ser. Adelaide			2	3		2		7
ser. Alachua †				1				1
ser. Bootle						1		1
ser. Bredeney †	1			1				2
ser. Carnac					2			2
ser. Cerro †		1	1	1	1			4
ser. Chester	1		1	1				3
ser. Choleraesuis var. Decatur				1				1
ser. Infantis		1			1			2
ser. Muenchen	1	2				2		5
ser. Orion	1			1				2
ser. Rottnest		1						1
ser. Waycross	1							1
S. enterica subsp. *diarizonae*								
(IIIb) ser. 35:k:z_{53} †	1							1
(IIIb) ser. 50:k:z_{35} ‡							1	1
S. enterica subsp. *salamae*								
(II) ser. 21:z_{10}:z_6	1	1	1					3
Total no. of animals tested	11	12	11	12	13	12	21	92
Animals positive	*7*	*6*	*4*	*8*	*4*	*5*	*1*	*35*
No. of isolations	*7*	*6*	*5*	*9*	*4*	*5*	*1*	*37*

† new *Salmonella* serovars isolated from *S. brachyurus* on Rottnest Island
‡ new *Salmonella* serovar isolated from *S. brachyurus* on the mainland
ser.= serovar
var.= variety

4.4.3 *Correlates of Salmonella with HMT, BLC and PBCM variables for Rottnest Island S. brachyurus*

Subtle differences in the HMT, BLC and PBCM datasets between *Salmonella* positive and negative animals were observed (SIMPER; Table 4-7a,b,c). This was evident in the WBC (less LYMPH, EOS, NEUT; and higher MONO), erythrogram (lower RBC, HGB, and PCV), and blood chemistry (higher ALP, BILT, CREAT, and UREA, lower GLUC, PROT and ALB) profiles of *Salmonella* positive animals. Though the differences in the HMT and BLC parameters between *Salmonella*-positive and *Salmonella*-negative animals were not significant (PERMANOVA: $p \geq 0.05$, Table 4-8a,b,c), the *p* values obtained were considered to be weak evidence of the absence of effect of *Salmonella* on these response variables.

Table 4-7 SIMPER analysis results indicating the contribution of specific variables to the observed differences in HMT (a), BLC (b), and PBCM (c) profiles of quokkas that were *Salmonella*-positive and *Salmonella*-negative on Rottnest Island. HMT= haematology, BLC= blood chemistry, PBCM= peripheral blood cell morphology.

		Taxon	Ct %	*Salmonella* +ve		*Salmonella* -ve	
				x̄	SD	x̄	SD
a.	HMT (31) † n +ve= 26 n -ve= 31	Lymphocytes (x10e9/L)	13.52	1.66	0.79	2.03	1.03
		RBC (x10e12/L)	11.98	5.60	0.92	5.85	0.78
		WBC (x10e9/L)	11.84	4.20	1.55	4.94	1.60
		Monocytes (x10e9/L)	11.76	0.07	0.04	0.06	0.06
		Basophils (x10e9/L)	11.72	0.02	0.02	0.02	0.02
		Eosinophils (x10e9/L)	11.01	0.32	0.28	0.51	0.49
		HGB (g/L)	9.978	105	16.4	108	12.3
		Neutrophils (x10e9/L)	9.652	2.13	0.97	2.32	1.12
		PCV (%)	8.544	32.7	4.43	33.5	4.79
b.	BLC (20.9) † n +ve= 30 n -ve = 36	Vit. E (mg/L)	9.55	6.89	1.74	6.44	1.63
		CK (U/L)	9.12	861	768	949	1,035
		PHOS (mmol/L)	8.83	1.01	0.34	1.2	0.46
		ALP (U/L)	7.96	11,278	15,617	5,433	3,308
		CHOL (mmol/L)	7.75	2.56	0.47	2.88	0.52
		GLUC (mmol/L)	7.40	4.20	2.01	4.27	2.71
		PROT (g/L)	7.14	60.2	3.67	60.8	5.59
		BILT (µmol/L)	6.91	5.01	2.05	4.39	1.47
		ALT (U/L)	6.87	222	76.7	222	62.4
		CALC (mmol/L)	6.77	2.24	0.18	2.19	0.23
		ALB (g/L)	6.49	36.1	1.44	36.3	1.97
		AST (U/L)	5.36	54.1	23.6	45.7	17.6
		CREAT (µmol/L)	5.12	74.1	17.9	69.4	15
		UREA (mmol/L)	4.75	7.10	1.87	6.88	1.67

				Salmonella +ve	*Salmonella* -ve	
			Ct %	Frequency (%)	Frequency (%)	OR ‡ (95% CI)
c.	PBCM (24.59) † +ve= 30 -ve= 36	Rouleaux formation	12.2	16 (53.3)	16 (44.4)	1.43 (0.54-3.78)
		Acanthocytes	12.1	17 (56.7)	19 (52.8)	1.17 (0.44-3.10)
		Heinz bodies	10.8	11 (36.7)	12 (33.3)	1.16 (0.42-3.20)
		Schistocytes	8.57	8 (26.7)	8 (22.2)	1.27 (0.41-3.93)
		Anisocytosis	8.18	26 (86.7)	27 (75)	2.17 (0.59-7.91)
		Keratocytes	8.12	7 (23.3)	8 (22.2)	1.07 (0.34-3.38)
		Flower Cells	8.01	4 (13.3)	10 (27.8)	0.40 (0.11-1.44)
		Echinocytes	7.80	6 (20)	8 (22.2)	0.88 (0.27-2.88)
		Poikilocytosis	6.61	25 (83.3)	31 (86.1)	0.81 (0.21-3.10)
		Hypochromasia	5.18	27 (90)	31 (86.1)	1.45 (0.32-6.65)
		nRBCs	4.84	27 (90)	32 (88.9)	1.13 (0.23-5.47)
		Polychromasia	3.87	28 (93.3)	33 (91.7)	1.27 (0.20-8.17)
		Howell-Jolly bodies	2.84	29 (96.7)	33 (91.7)	2.64 (0.26-26.7)
		Reactive lymphocytes	0.90	30 (100)	35 (97.2)	2.58 (0.10-65.6) *

Ct: Percent of contribution to difference
† Overall average dissimilarity
‡ Odds ratio for the presence of the taxon in *Salmonella* +ve individuals and 95% confidence intervals calculated using Woolf's method (1955)
* calculated by adding 0.5 to each observed value (Altman 1999)

There was no particular clustering of the variables (HMT, BLC and PBCM) either across seasons (nMDS analyses: Figure 4-6a,c,e) or across sexes (Figure 4-6b,d,f). Contrary, there was a consistent and significant effect (PERMANOVA: $p < 0.01$; Table 4-8) of season and sex on BLC profiles, whereas season was the only factor to have a significant effect ($p < 0.01$) on PBCM of quokkas on Rottnest Island. No clustering was evident in their respective nMDS plots (Figure 4-6c,d,e,f).

Table 4-8 Two-way PERMANOVA of selected HMT variables (a) (corrected WBC, RBC, HGB, PCV, and absolute counts for leukocytes obtained with a manual differential on a blood smear), BLC analytes (b) (ALP, ALT, AST, CK, PROT, ALB, CALC, PHOSP, CHOL, BILT, GLUC, CREAT, UREA and Vitamin E), and PBCM (c), for *S. brachyurus* positive and negative to <u>any *Salmonella* serovar</u>, with season and sex as independent factors. Bray-Curtis similarity index, Permutations N= 9,999. Only two independent factors could be tested simultaneously, and therefore the presence of *Salmonella* was tested first with season, and then secondly with sex of the animal. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

		Data	Factor	SS	df	MS	*F*	*p*
a.	All *Salmonella* serovars	HMT	Factor *Salmonella*	0.108	1	0.108	1.31	0.08 †
			Factor Season	0.179	3	0.060	0.724	0.39
			Interaction	-1.24	3	-0.414	-5.03	0.82
			Residual	4.03	49	0.082		
			Total	3.08	56			
			Factor *Salmonella*	0.108	1	0.108	1.90	0.07 †
			Factor Sex	0.061	1	0.061	1.08	0.34
			Interaction	-0.111	1	-0.111	-1.95	0.45
			Residual	3.02	53	0.057		
			Total	3.08	56			
b.		BLC	Factor *Salmonella*	0.039	1	0.039	1.46	0.05 ‡
			Factor Season	0.215	3	0.072	2.70	**0.01**
			Interaction	-0.320	3	-0.107	-4.02	0.25
			Residual	1.54	58	0.027		
			Total	1.47	65			
			Factor *Salmonella*	0.039	1	0.039	1.69	0.07 ‡
			Factor Sex	0.063	1	0.063	2.75	**0.01**
			Interaction	-0.05	1	-0.05	-2.20	0.34
			Residual	1.42	62	0.023		
			Total	1.47	65			
c.		PBCM	Factor *Salmonella*	0.046	1	0.046	0.898	0.36
			Factor Season	0.409	3	0.136	2.678	**0.01**
			Interaction	-0.752	3	-0.251	-4.93	0.95
			Residual	2.95	58	0.051		
			Total	2.65	65			
			Factor *Salmonella*	0.015	1	0.015	0.340	0.80
			Factor Sex	0.089	1	0.089	1.98	0.09
			Interaction	-0.252	1	-0.252	-5.60	0.99
			Residual	2.80	62	0.045		
			Total	2.65	65			

SS: sum of squares
Df: degrees of freedom
MS: mean sum of squares
† Effect size for haematology $d_{Cohen's}$= 0.34 (moderate)
‡ Effect size for blood chemistry $d_{Cohen's}$= 0.32 (moderate)

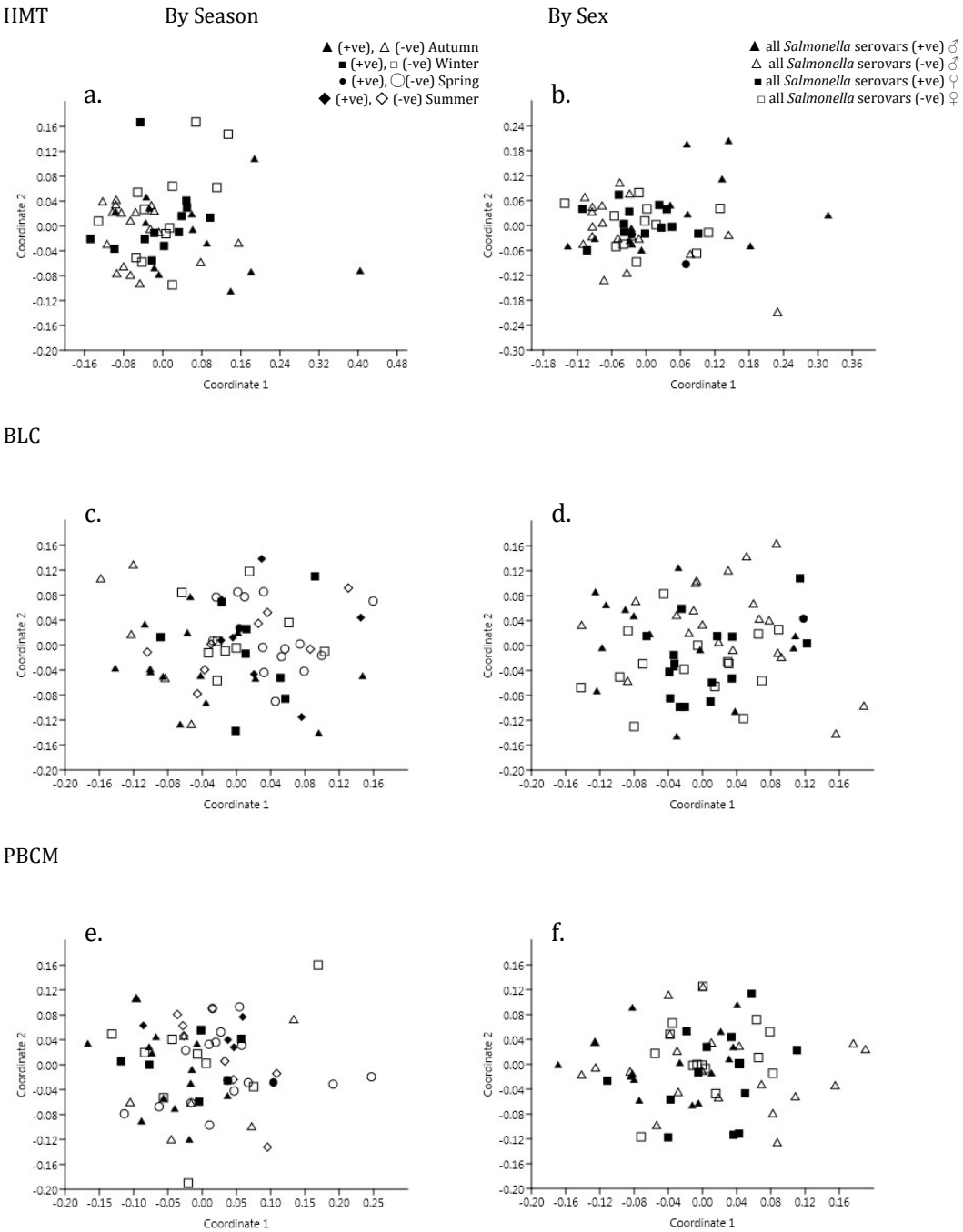


Figure 4-6 Non-metric MDS plots with Bray-Curtis similarity measure, illustrating the structural dissimilarity of HMT (a, b: stress statistic= 0.158), BLC (c. stress statistic= 0.273; d. stress statistic= 0.238), and PBCM communities (e, f: stress statistic= 0.245) in *Salmonella*-positive and *Salmonella*-negative animals on Rottnest Island. Covariates: season for left hand side plots, sex for right hand side plots. Key legends should be read for the left hand and right hand panels separately. Note that the distances along the axes are unit-less, therefore the positions of the points in the plots are relative distances from one another rather than absolute differences read in these units. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

4.4.4 *Relation between Salmonella spp. and: (i) physical examination findings, (ii) other organisms*

There were no differences in the physical examination findings between animals positive or negative for *Salmonella* (Table 4-9). There was a significant (p= 0.012) association between the presence of microfilariae in peripheral blood between *Salmonella*-positive and *Salmonella*-negative animals, with a greater proportion of microfilariae-positive animals in the *Salmonella*-positive group (Table 4-10). Furthermore, the probability of microfilariae-positive animals to be positive to *Salmonella* was 3.88 (95%, CI 1.31-11.5) times higher than microfilariae-negative animals. Within the *Salmonella* sampled group, microfilariae-positive animals had a significantly ($\bar{x}$= 3.901, $var(X)$= 1.46, n= 22) lower WBC than microfilariae-negative animals ($\bar{x}$= 4.651, $var(X)$= 3.28, n= 45); t(58.6)= 2; p= 0.044. Similarly, WBC was significantly lower in *Salmonella*-positive animals ($\bar{x}$= 3.925, $var(X)$= 2.41, n= 31) than in *Salmonella*-negative animals ($\bar{x}$= 4.817, $var(X)$= 2.80, n= 36); t(64.6)= 2.3; p= 0.027. There were no other significant associations between the presence of other organisms (i.e. MaHV-6, *Cryptococcus* spp., *Theileria* sp., *Babesia* sp., nematode eggs or *Eimeria* oocysts) with the presence of *Salmonella* spp. (Table 4-10).

Table 4-9 Associations between physical examination findings and the presence of *Salmonella* in animals from Rottnest Island. OR= odds ratio, CI= confidence interval, CRT= capillary refill time, MM= mucous membranes, EP= external parasites.

	Salmonella spp.			
Finding	+ve (%) n=34	-ve (%) n=37	OR † (95% CI)	χ^2 1, n= 71
Abnormal mentation [a]	1 (1.41)	0 (0)	3.36 (0.13-85.3) *	= 1.10, p= 0.293
Abnormal CRT [b]	4 (5.63)	2 (2.81)	2.33 (0.40-13.6)	= 0.92, p= 0.335
Abnormal MM [c]	10 (14.08)	10 (14.08)	1.13 (0.40-3.17)	= 0.05, p= 0.823
Cloudy eye	1 (1.41)	1 (1.41)	1.09 (0.07-18.1)	= 0.43, p= 0.511
Dehydration [d]	16 (22.53)	17 (23.94)	1.05 (0.41-2.66)	= 0.01, p= 0.925
Ear notches	5 (7.04)	10 (14.14)	0.47 (0.14-1.54)	= 1.61, p= 0.203
Flaky skin	1 (1.41)	0 (0)	3.36 (0.13-85.3) *	= 0.01, p= 0.966
Fur loss	11 (15.49)	6 (8.45)	2.47 (0.80-7.66)	= 2.53, p= 0.111
Presence of EP	9 (12.67)	5 (7.04)	2.30 (0.69-7.74)	= 1.88, p= 0.170
Skin erosions	2 (2.82)	1 (1.41)	2.25 (0.19-26.0)	= 0.01, p= 0.940
Wheezes	0 (0)	1 (1.41)	0.35 (0.01-8.95) *	= 0.01, p= 0.966

[a] Diminished response to external stimuli
[b] Greater than two seconds
[c] Pale and blue tinted mucous membranes
[d] Skin tenting for longer than 2 seconds
† Odds ratio for the presence of the taxon in *Salmonella* +ve individuals and 95% confidence intervals calculated using Woolf's method (1955)
* calculated by adding 0.5 to each observed value (Altman 1999)

Table 4-10 Results for associations of other infectious agents occurring concomitantly in animals from Rottnest Island tested for *Salmonella.* OR= odds ratio, CI= confidence interval, MaHV-6= Macropodid herpesvirus 6.

	Salmonella spp.			
Finding	+ve/n (%)	-ve/n (%)	OR † (95% CI)	Statistics
Babesia sp. [a]	1/26 (3.84)	0/33 (-)	3.94 (0.15-100.8) *	$\chi^2_{1, n=59} = 0.01$, $p = 0.904$
Cryptococcus spp. [b]	4/29 (13.8)	1/31 (3.22)	4.80 (0.5-45.7)	$\chi^2_{1, n=60} = 1.03$, $p = 0.311$
Eimeria spp. [c]	24/32 (75)	26/31 (83.8)	0.58 (0.17-2.01)	$\chi^2_{1, n=63} = 0.75$, $p = 0.384$
MaHV-6 [a]	3/26 (11.5)	2/33 (6.06)	2.02 (0.31-13.1)	$\chi^2_{1, n=59} = 0.08$, $p = 0.780$
Microfilariae [c]	15/31 (48.3)	7/36 (19.4)	**3.88 (1.31-11.5)**	$\chi^2_{1, n=67} = 6.33$, $\mathbf{p = 0.012}$ ‡
Nematode eggs [c]	29/32 (90.6)	27/31 (87.1)	1.43 (0.29-7.0)	$\chi^2_{1, n=63} = 0.01$, $p = 0.964$
Theileria sp. [a]	5/26 (19.2)	5/33 (15.1)	1.33 (0.34-5.21)	$\chi^2_{1, n=59} = 0.17$, $p = 0.678$

[a] Screened by PCR [MaHV-6: see Chapter 6, section 6.3.1; Piroplasms: Chapter 7, section 7.3.1.1]
[b] Screened by culture methods, isolates characterised by PCR (see Chapter 5, section 5.3.1)
[c] Screened by light microscopy [gastrointestinal parasites: see Chapter 3, section 3.4.1; microfilariae: see Chapter 7, section 7.3.1]
† Odds ratio for the presence of the taxon in *Salmonella* +ve individuals and 95% confidence intervals calculated using Woolf's method (1955)
‡ Effect Size $d_{Cohen's} = 0.63$ (moderate)
* calculated by adding 0.5 to each observed value (Altman 1999)

4.5 Discussion

Free-ranging *S. brachyurus* from Rottnest Island and the mainland of WA were screened for *Salmonella* (faecal pellets collected by rectal palpation) by culture methods and isolates were identified to the serovar level by serotyping. There was a subtle effect of *Salmonella* spp. on the HMT and BLC response variables of infected animals. By contrast, there was no association between on-site physical examination findings and the presence of *Salmonella* spp. Concomitant infection with microfilariae was associated with a greater probability (OR 3.88, CI 1.31-11.50; $p = 0.012$) of being infected with *Salmonella*, *Salmonella* infected animals that were also positive to microfilariae had lower WBC than *Salmonella*-positive animals that were negative to microfilariae ($p = 0.042$). Sixteen serovars were recovered, of which the most prevalent ones were *S. enterica* ser. Adelaide and ser. Muenchen, followed by ser. Cerro. Of all the serovars recovered, five were new unreported ones in the quokka. Overall, there was a greater prevalence of *Salmonella* on Rottnest Island (prevalence $^{n=71}$ =47.9%, CI 36.5-59.4) than on the mainland (prevalence $^{n=21}$ =4.8%, CI 0.8-22.7), with the highest prevalence at Serpentine (66.7%, CI 39.1-86.2) and Barker Swamp (63.6%, CI 35.4-84.8) sites, while quokkas from the Settlement site had the lowest prevalence (30.8%, CI 12.7-57.6). This prevalence was found to have a seasonal pattern, with the lowest and highest prevalences happening in winter and summer respectively.

The combined prevalence of *Salmonella* obtained in this study for animals on the mainland (4.8%, 1/21), is considerably lower than that previously reported (63.6%) for animals at

Byford (~40 km South of Perth) and Holyoake (~100 km S.S.E. of Perth) (Hart 1980 ; Hart, Bradshaw, & Iveson 1986). However, because of inconsistencies in the sampling techniques, any of the isolations obtained from mainland animals in these earlier studies cannot be linked back to the animals with acceptable certainty given the risk of cross-contamination. *S. enterica* ser. Orientalis and ser. Newbrunswick were isolated at Byford, however, faecal samples were collected from the ground (Hart 1980). Two different serovars were isolated from animals at Holyoake, *S. enterica* ser. Muenchen from nine animals, and ser. Newington from eight animals, however, these results were not just obtained from rectal swabs but also faecal pellets collected from the floor of the traps. Additionally, animals were manipulated with equipment and holding bags that have been previously used on Rottnest Island (Hart 1980), where these two serovars appear to be among the most prevalent (Iveson & Hart 1983). In consequence, these isolations cannot provide an indication of the presence of *Salmonella* in *S. brachyurus*. Although cross-contamination of mainland samples may have been possible in this study, we believe there was a lower risk of it considering that faecal pellets were collected directly from the animal by rectal palpation after having disinfected the pericloacal region. Additionally, samples were considered not viable if they have been in contact with any other surfaces than those of the examination gloves and the cloacal lining. Furthermore, all handling equipment (e.g. restraining bags) used on mainland animals was exclusive for mainland fieldwork, and all medical equipment (e.g. anaesthetic equipment) was chemically disinfected with F10SCXD (Health and Hygiene Pty. Ltd, Florida Hills, South Africa) between trapping sessions.

The prevalence of *Salmonella* on the mainland (i.e. 4.5%) detected in this study, is within the prevalences reported by previous studies for Australian native wildlife. For instance, Parsons et al (2010) obtained prevalences ranging from 2% (in macropods and other Dasyuromorphia and Diprotodontia species) to 9% in snakes and lizards, while Potter et al (2011) obtained a prevalence of 3.6% in western grey kangaroos. Similarly, studies outside Australia have also determined similar low prevalences of *Salmonella*, ranging from 1% in white tailed deer (*Odocoileus virginianus*) (Renter *et al.* 2006) to 9.23% in the beech marten (*Martes foina*) (Nowakiewicz *et al.* 2016). These results however, should be interpreted with caution. Given that infected animals can intermittently shed *Salmonella* in their faeces (Sanchez *et al.* 2002 ; Spier *et al.* 1991), it is possible that negative mainland quokkas were in fact positive but not shedding *Salmonella* at the time of sampling, in which case prevalence would have been higher. Similarly, mainland quokkas might have been sampled during the time of the year (i.e. Autumn and Spring) when *Salmonella* proliferation was not at its peak, hence obtaining a low prevalence. Previous studies in

various species such as opossums (Kourany & Vasquez 1975 ; Ruiz-Piña *et al.* 2002) have observed a greater peak of *Salmonella* isolations during the dry season. This coincides with our findings on Rottnest Island where there was a greater prevalence of *Salmonella* during summer (see Figure 4-4), which has also been previously shown by Iveson et al. (2007). Lastly, *S. brachyurus* in the mainland is a very elusive animal and difficult to trap. Hence, given the small number of animals captured and processed, it is possible that positive animals may have not been included in the sample. Nevertheless, our data still retains its reliability given that our results can be linked-back to the animals with a greater degree of certainty.

The estimated prevalence of *Salmonella* on Rottnest Island obtained in this study differed from previous studies in that, it was lower than the first published prevalence back in 1973 (71%, 62/87) (Iveson & Bradshaw 1973) but higher than subsequent studies: 38.4% (1,551/4,038) by Iveson and Hart (1983) [9] and 32% (197/621) by Iveson et al. (2007) [10]. This difference may be explained by the type of samples that were used in each study, but also by the sample size. Hart et al. (1985) determined that faecal pellets were more effective than rectal swabs when detecting *Salmonella* at low concentrations (below 10^3 of *Salmonella* per gram of faeces) in *S. brachyurus* [11]. Methodologies used for the highest prevalences reported (i.e. 47.9% and 71%) both had a sample size smaller than 100 animals and used faecal pellets, with the latter also using rectal swabs in duplicate. In contrast, lower prevalences (i.e. 38.4% and 32%) were obtained when animals were screened with rectal swabs only, and the sample size ranged from 621-4,038 animals. It might be reasonable to infer then, that by screening for *Salmonella* on faecal pellets, faecal *Salmonella* loads that are usually not detected with rectal swabs would have been detected, hence a higher number of positive animals. However, using faecal pellets only (collected by rectal palpation) may increase the likelihood of not screening possible positive animals if there was no rectal content. Consequently, a higher prevalence and serovar richness could have been obtained on Rottnest Island in this study by using faecal pellets in combination with rectal swabs (when a faecal pellet could not be retrieved).

In both cases (Rottnest Island and mainland), diagnostic methods with a greater sensitivity such as PCR may have been helpful in detecting low levels of *Salmonella*, and consequently detecting a greater prevalence. However, there is no consensus in the literature as to whether molecular methods are more effective in detecting *Salmonella* than the current gold standard (culturing). For instance, Eriksson and Aspan (2007) when

[9] Data for this paper was collected over a period of 10 years, between 1972 and 1983
[10] Data for this paper was collected over the period of March 1984 and March 1985
[11] Above 10^3 *Salmonella* per gram of faeces the efficiency in obtaining *Salmonella* on culture media was similar for both type of samples (rectal swabs and faecal pellets)

screening for *Salmonella* in artificially contaminated faecal samples from cattle, pigs, and poultry, concluded that microbiological methods were more reliable and sensitive, and that PCR-based methods proved almost as sensitive and specific. Myint et al. (2006) obtained the same *Salmonella* prevalence when testing both techniques on the same poultry meat samples. Similarly, Wilkins et al. (2010) concluded that Real Time Polymerase Chain Reaction (RT-PCR) performed similarly to culture when screening for *Salmonella* in the faeces of grow–finish pigs. By contrast, other authors indicate that PCR-based methods are more sensitive and detect *Salmonella* more efficiently than culture techniques (Amavisit *et al.* 2001 ; Kumar, Surendran, & Thampuran 2008). Though quicker results can be obtained through PCR-based methods (Eriksson & Aspan 2007 ; Khan *et al.* 2014 ; Myint *et al.* 2006) and in some cases with a greater degree of sensitivity, from an epidemiological stand point, PCR-methods have not and cannot reliably replace culture methods and serotyping (Davies 2013 ; Koyuncu, Andersson, & Haggblom 2010 ; Wattiau, Boland, & Bertrand 2011), given that less than 10% of today's known serovars can be identified to the serovar level by PCR-methods. Even if both methods are combined (cultural and molecular) matching a positive molecular result to a positive culture result and a given serovar presents as a difficult and inconvenient task. Although we might have not detected high numbers of *Salmonella* we believe our data is epidemiologically valuable. Nevertheless, the estimated prevalence of *Salmonella* in our study may be an underestimation of the true prevalence of the bacteria on Rottnest Island and the mainland.

A seasonal pattern of *Salmonella* infections on Rottnest Island was evident. Similar observations have been previously documented in the Rottnest Island quokka (Iveson & Bradshaw 1973 ; Iveson, Bradshaw, & Hart 2007 ; Iveson & Hart 1983), in which more animals shedding *Salmonella* were detected during the dry season (see Figure 4-4). Increased *Salmonella* shedding by infected animals (Ruiz-Piña *et al.* 2002), as well as increased serovar diversity (Haley, Cole, & Lipp 2009) have been linked with the dry. Our findings also indicate that the previously reported association between settled areas and a low prevalence of *Salmonella* in quokkas (Hart, Bradshaw, & Iveson 1985 ; Iveson, Bradshaw, & Hart 2007 ; Iveson & Hart 1983) may still persist. We observed a lower prevalence of infection in heavily human-intervened sites (i.e. Settlement: 30.77%, n= 13), while a higher prevalence was observed in less human-intervened sites (i.e. Serpentine: 66.66%, n= 12, Barker Swamp: 63.63%, n= 11). These different rates of infection were attributed by the authors (Hart 1980 ; Hart, Bradshaw, & Iveson 1985) to the year-round supplementary feeding that animals on the Settlement were receiving, from having access to the rubbish disposal site and from visitors.

Previous studies have suggested female quokkas may be more susceptible to infection with *Salmonella* than males. For instance, according to Hart (1980) "female animals are more rapidly affected than males". While Iveson et al. (2007) reported a significantly (p= 0.037) higher prevalence (39.9%, 63/158) of *Salmonella* in females than in males (27.5%, 36/131), captured at the lighthouse swamp (~1 km west of the nearest site sampled in this project: Barker Swamp), although this was not discussed. In our study, although the probability of being positive to *Salmonella* was 1.30 times greater in females than in males, this difference was not significant (p= 0.581). This suggests that the probability of *Salmonella* infection is similar for males and females. However, this may be the result of a small sample size [for instance compared to that of Iveson et al. (2007)]. In this context, it is worth noting that the prevalence of *Salmonella* in quokkas on Rottnest Island peaks between November and March (based on our data), which happens to coincide with the female's gestation, births and lactation events in *S. brachyurus* (Shield 1964). Studies on mammals, including humans, have shown that females may be naturally more susceptible to disease due to the burden of the metabolic demands of pregnancy. For instance, Pejcic-Karapetrovic (2007), found abortion rates of ~100% and host fatality of >60% in strains of pregnant mice, that are normally resistant to *S. enterica* ser. Typhimurium, while increased faecal shedding of *Salmonella* has been observed in pregnant heifers, as a result of pregnancy-related immunosuppression (Anderson *et al.* 2001). Although pregnancy-associated immunosuppression has not been studied in macropods, it has been linked to an increase in host susceptibility to infectious diseases in other species, and may also be involved in the increased infection rate of the female *S. brachyurus* on Rottnest Island, as reported by Iveson et al (2007).

Generally, animals in this study were apparently healthy, with no obvious signs of clinical salmonellosis, and the physical findings we observed had no association with the presence of *Salmonella* (see Table 4-9). Similarly, *Salmonella* was found to have no effect on the HMT, BLC or PBCM of quokkas on Rottnest Island. However, we advise caution when interpreting these results. Considering that the p values we obtained for the HMT and BLC analyses were close (i.e. 0.07-0.08) or on the statistical cut-off (i.e. 0.05), these were considered as being insufficient evidence to conclude that *Salmonella* has no effect on the HMT and BLC parameters of *Salmonella*-infected quokkas. It appears the study design was not able to separate asymptomatic carriers from animals with active disease, which may imply that subclinical carriage of *Salmonella* may be more common than active salmonellosis. However, this may be attributable to a small sample size and not to the absence of disease. However, close examination of the HMT and BLC data revealed different profiles between *Salmonella*-positive and -negative animals (see Table 4-7), with

lower RBC, PCV, HGB, WBC, LYMPH, NEUT, and EOS, and higher MONO in positive animals when compared to those that were negative. Similarly, differences were present in their BLC, with positive animals having higher values for BILT, ALP, AST, CREAT and UREA, and lower values for GLUC, PROT, and ALB, than those of negative animals. With the exception of PCV, RBC, and HGB that may be attributable to the seasonal anaemia experienced by quokkas on Rottnest Island (Barker *et al.* 1974 ; Shield 1971), and CALC concentrations in serum, the overall trend observed in all other parameters (i.e. HMT and BLC) between *Salmonella*-positive -negative quokkas resembles in great degree, the HMT and BLC of calves with clinical salmonellosis due to *S. enterica* ser. Typhimurium (Santos *et al.* 2002).

Similarly, despite the absence of statistical analysis of the HMT, BLC, PBMC and physical examination data of the mainland cohort (given that only one animal was found positive), our field observations of the mainland quokka captured in the vicinity of Jarrahdale (~45 km south-east of Perth), from which *S. enterica* subsp. *diarizonae* (IIIb) ser. 50:k:z35 was isolated, did not resemble any of the known forms of disease caused by *Salmonella*. The negative results for *S. enterica* subsp. *enterica* serovars from mainland samples, could suggest that mainland animals may be immunologically naïve to serovars of the subsp. *enterica*, or alternatively, that mainland animals are able to mount a competent immunological response capable of controlling *Salmonella* proliferation. However, the immunologically naïve scenario, may explain the mortalities Hart (1980) reported and attributed to *Salmonella*, with quokkas dying acutely sometime after being in contact with contaminated equipment that had previously being used on Rottnest Island. This scenario may also support that serovars Muenchen and Newington recovered from quokkas on the mainland (Hart 1980), were in fact the result of cross contamination (Rottnest Island equipment) and not an indication of the presence of these serovars on the mainland.

Concomitant infection with microfilariae was found to be positively associated with a greater probability of being *Salmonella* positive, with microfilaria-positive animals being 3.88 times (95% CI 1.31-11.50) more likely to be infected with *Salmonella* than negative animals. Although further studies would be necessary to understand the relation between microfilariae-*Salmonella* and the immune system of the Rottnest Island quokka, both organisms were associated (p= 0.044) with lower WBC in infected animals than in uninfected ones. This suggests that there may be a synergistic interaction of these organisms on the immune system of the host. However, it is generally accepted (with some exceptions) that in helminth-microparasite (e.g. bacteria) infections, helminths appear to impair the immune response of the host (Bordes & Morand 2011). In vivo and in vitro studies, in humans for the most part, have shown that individuals with

microfilarial infection (or exposed to microfilarial lysate) experience reduced expression of toll-like receptor 4 (TLR4) mRNA, interleukin 12 (IL-12) and 8 (IL-8), as well as interferon gamma (IFN-γ), in conjunction with impairment of Myeloid differentiation primary response 88 (MyD88) signalling, (Semnani *et al.* 2006 ; Semnani *et al.* 2008), suppression of the proliferation of CD4+ T cells, and an increased expression of IL-10, a potent inhibitor of cellular response (Moore *et al.* 1993 ; O'Regan *et al.* 2014). Interleukin 10 has been linked to increased susceptibility in mice to infection with *Salmonella* (Su *et al.* 2014). Moreover, circulating microfilariae appear to have a suppressive effect on the production of B lymphocytes, particularly B-1 cells (Mishra *et al.* 2014), which has been correlated to increased mortality after infection with bacteria (e.g. *S. enterica* ser. Typhimurium and *Streptococcus pneumoniae*) (Briles *et al.* 1981 ; O'Brien *et al.* 1979) and viruses (e.g. influenza virus and vesicular stomatitis virus) (Baumgarth *et al.* 1999 ; Ochsenbein *et al.* 1999). The significantly lower WBC in both, *Salmonella*-positive and microfilariae-positive animals when compared to their corresponding negative groups, may indicate that both organisms are related to some degree of immunosuppression of the host. Consequently, it may be possible that quokkas on the island may not be able to mount an adequate immune response, to recognise, control and resolve the infection, which may explain the characteristic chronicity of *Salmonella* infections on Rottnest Island.

From a conservation perspective, it is difficult to predict whether *Salmonella* infections could become a threatening process to the Rottnest Island quokka or not. Nevertheless, the apparent association between microfilarial infection and low WBC we observed, suggests that quokkas on Rottnest Island may be in a state of impaired immune response. This, coupled with chronic exposure to stress through changes in their ecosystem (e.g. habitat clearing, increasing visitation) could gradually aggravate the state of immunosuppression. This in turn, could make the Rottnest Island quokka more susceptible to other infectious organisms. From a public health point of view, tracking the incidence, prevalence and abundance of *S. enterica* subsp. *enterica* serovars on Rottnest Island is recommended for adequate public health management.

5. Isolation of *Cryptococcus neoformans* var. *grubii* (serotype A) and *C. magnus* from the nasal lining of free-ranging quokkas on Rottnest Island

STATEMENT OF AUTHOR CONTRIBUTION

P. Martinez-Perez: designed the general scope and structure of the chapter, initiated the research, carried out all fieldwork procedures, carried out all the laboratory procedures for the isolation and biochemical and molecular characterisation of cryptococcal isolates and other yeasts, performed blood smear assessment, entered and analysed the data, and wrote the chapter.

P.A. Fleming: advised and assisted with statistical analyses, and provided editorial comments to versions of the chapter from draft to final version.

T.H. Hyndman: provided editorial comments to versions of the chapter from draft to final version.

K. Townsend: provided training in culturing techniques.

Murdoch University Veterinary Hospital, PathWest and VetPath: carried out preliminary screening (culturing) of 34 swabs.

5.1 Abstract

Cryptococcus species are environmental basidiomycetous yeasts, with a worldwide distribution and remarkable environmental adaptation. Although many species do not cause disease, *Cryptococcus. neoformans* and *Cryptococcus. gattii* are the causative agents of cryptococcosis, a life-threatening infection and a significant public health problem worldwide. Infection affects especially immunocompromised individuals, both animals and humans alike. In wildlife, cryptococcosis appears to be more prevalent in captive populations. Using cultural and molecular methods, we studied yeasts isolated from nasal swabs collected from 130 free-ranging quokkas (*Setonix brachyurus*) on Rottnest Island (Rottnest Island, n= 97) and the mainland (ML, n= 33) of Western Australia (WA). Cryptococcal isolates [*C. neoformans* var. *grubii* (serotype A), prevalence= 2.1% 95% CI 0.4-6.4; *C. magnus*, prevalence=1.03%, CI 0.1-4.7; and four unspeciated *Cryptococcus* spp.,

prevalence= 4.12%, CI 1.4-9.5] were recovered from the nasal lining of apparently healthy *S. brachyurus* on Rottnest Island, but not on the mainland. Both *C. neoformans* var. *grubii* and *C. magnus*, were isolated from animals captured at human-populated areas on Rottnest Island. There was no significant association of the presence of cryptococcal organisms in the nasal lining of *S. brachyurus* with variations in their haematology (HMT), blood chemistry (BLC) or peripheral blood cell morphologies (PBCM). To the best of our knowledge, this is the first documented isolation of *C. neoformans* var. *grubii* (serotype A) and *C. magnus* in a free-ranging macropod in WA. The recovery of *C. neoformans* var. *grubii* from quokkas suggests the presence of an environmental source on Rottnest Island, and constitutes an important finding for public health and management of wild and captive *S. brachyurus* These findings suggest that *S. brachyurus* is an asymptomatic carrier of *Cryptococcus* spp.

5.2 Introduction

Cryptococcus species are environmental basidiomycetous yeasts, with a worldwide distribution and remarkable environmental adaptation, with some species surviving high salinity, extreme cold temperatures (e.g. Antarctica), extreme altitudes (e.g. Himalayas) (Casadevall & Perfect 1998) and thriving in rich ionising radiation environments (Dadachova *et al.* 2007). Many cryptococcal species (e.g. *C. albidus, C. laurentii, C. uniguttulatus*, and *C. magnus*) are usually considered to be saprophytes that rarely cause disease. Only a few species are known to cause disease; of which, the most important are *Cryptococcus neoformans* var. *grubii*, *C. neoformans* var. *neoformans*, and *C. gattii* (Casadevall & Perfect 1998 ; Fothergill 2007 ; Sorrell *et al.* 1996 ; Sykes & Malik 2012).

Cryptococcus neoformans and *C. gattii* have different environmental niches, and the disease for which they are responsible (i.e. cryptococcosis), has different epidemiological characteristics. *Cryptococcus gattii* is primarily associated with *Eucalyptus* spp. trees, usually causing infection in the immunocompetent host (Cafarchia 2012). By contrast, *C. neoformans* var. *neoformans* (serotype D) and var. *grubii* (serotype A) are associated with disease in individuals with defective cell mediated defences (Del Poeta & Casadevall 2012 ; Mitchell & Perfect 1995) and are found worldwide in close relation with excreta of birds (Orders Psittaciformes, Passeriformes, Columbiformes and Falconiformes) (Cafarchia 2012), although Columbiformes are the ones most commonly associated with *C. neoformans* (Sykes & Malik 2012). Serotypes A and D have also been isolated from soil contaminated with bird excreta (Casadevall & Perfect 1998), and from material of a variety of plant species (Granados & Castañeda 2005 ; Nishikawa *et al.* 2003 ; Pfeiffer &

Ellis 1993). *C. neoformans* var. *grubii* is commonly isolated from human clinical cases and a wide variety of environmental samples (Cogliati 2013 ; Li *et al.* 2012 ; Meyer *et al.* 2003 ; Nishikawa *et al.* 2003 ; Steenbergen & Casadevall 2000). In Australia, var. *grubii* is the most common isolate from clinical samples of animals and humans, and is strongly associated with immunocompromised patients (Chen *et al.* 2000 ; Payne *et al.* 2005 ; Sykes & Malik 2012), but has also been observed in immunocompetent dogs and cats (Krockenberger *et al.* 2005).

Significantly less prevalent, *C. magnus* has been isolated from plants and soil (Korhola *et al.* 2014 ; Nagornaya *et al.* 2003 ; Sipiczki 2013), e.g. mangrove sediment GenBank Accession No. KJ706585), invertebrates (German cockroach *Blatella germanica*) (Zheltikova, Glushakova, & Alesho 2011), and apparently healthy domestic cats and wild birds (Table 5-1 and references therein). Although *C. magnus* is generally considered to be saprophytic fungus with little or no medical significance, it has also been recovered from children with leukaemia (Khan, Mokaddas, *et al.* 2011) and in recent decades has emerged (together with other species; e.g. *C. uniguttulatus*, *C. laurentii*, and *C. albidus*) as a cause of clinical disease in immunocompromised hosts (Danesi *et al.* 2014).

Serological and culturing studies indicate that *Cryptococcus* can be carried asymptomatically (Connolly *et al.* 1999 ; Krockenberger *et al.* 2002 ; Malik *et al.* 1997); however, conditions that put pressure on the immune system (e.g. drought, availability of food, other infections) may increase the incidence of infection (Krockenberger *et al.* 2005). Cryptococcosis in wildlife is likely to be associated mainly with exposure to excessive numbers of basidiospores; although the mechanism of infection has not yet been established, direct inhalation of environmental basidiospores is the most likely infection route (Danesi *et al.* 2014 ; Sykes & Malik 2012). These inhaled organisms can then become transient, or proceed to colonise tissues and form primary foci of infection in the nasal cavity, paranasal sinuses or lung parenchyma (Connolly *et al.* 1999 ; Sykes & Malik 2012). Once infection is established, it is believed the yeast spreads to other organs either through the cribriform plate or haematogenously via macrophages, with particular tropism for the central nervous system (Connolly *et al.* 1999 ; Sykes & Malik 2012).

Although cryptococcosis can have a localised presentation, it is usually a systemic disease with important global medical significance that affects humans and a wide range of animal species (Del Poeta & Casadevall 2012 ; Sykes & Malik 2012), including reptiles, birds, eutherians, and marsupials (Table 5-1 and references therein); cases in poikilothermic species are very rare (Miller *et al.* 2004). According to Park et al. (2009), in humans, *C.*

neoformans has been responsible for an estimated 1 million cases of cryptococcal meningitis per year, resulting in approximately 625,000 deaths. In WA, *C. gattii* has been suggested to be the most common causative agent of cryptococcal disease in domestic animals (McGill *et al.* 2009), but in studies across the rest of Australia, *C. neoformans* (primarily var. *grubii*) appears to be more frequent in domestic animals (particularly cats and dogs) (Sykes & Malik 2012), while *C. gattii* is more prevalent in wildlife (Krockenberger *et al.* 2005). Cryptococcosis due to *C. magnus* appears to be infrequent, and has only been associated with one confirmed case of multisystemic disease in a cat (Poth *et al.* 2010). To the best of our knowledge there are no records indicating the detection of this yeast from wildlife.

In wildlife, *C. neoformans* and *C. gattii* have mostly been reported in captive animals (Table 5-1). Of these reports, the majority of cryptococcal infections and isolations have been described in koalas (*Phascolarctos cinereus*) (Table 5-1 and references therein). Both *C. neoformans* var. *grubii* and *C. gattii* have been isolated from the nasal mucosa of koalas, but only *C. gattii* has been recognised as a cause of clinical infection (Payne *et al.* 2005). Interestingly, cultural studies of nasal swabs from captive koalas at the Kanazawa Zoological Gardens, Japan, yielded a greater prevalence of *C. neoformans* than *C. gattii* (Kido *et al.* 2012).

Table 5-1 Summary of studies that have isolated *Cryptococcus neoformans* (var. *grubii* and var. *neoformans*) and *C. gattii* from wildlife species. No cases of *C. magnus* as a primary agent of disease in Australian wildlife were found in the literature; (number of cases). C= captive, FR= free-ranging.

Wildlife host species	C/FR	Isolate	Observations	Reference
Brushtail possum (*Trichosurus vulpecula*),	C	n.s.	n.s.	(Ladds 2009)
Cheetah (*Acinonyx jubatus*)	C	n.s.	pulmonary disease and meningoencephalomyelitis	(Berry, Jardine, & Espie 1997)
Common wombat (*Vombatus ursinus*)	C	n.s.	n.s.	(Ladds 2009)
Gilbert's potoroo (*Potorous gilbertii*) and long-nose potoroo (*P. tridactylus*)	C	n.s.	cryptococcosis	(Vaughan *et al.* 2007)
Koala (*Phascolarctos cinereus*)	C and FR	*C. neoformans* and/or *C. gattii* [a]	apparently healthy, cryptococcal meningoencephalitis, pneumonia, nasal and nasopharyngeal disease, lymphadenopathy	(Connolly *et al.* 1999 ; Krockenberger, Canfield, & Malik 2002, 2003 ; Krockenberger *et al.* 2005 ; Ladds 2009)
	C	*C. neoformans* and *C. gattii* [b]	n.s.	(Kido *et al.* 2012)
Parma wallaby (*M. parma*)	C	n.s.	n.s.	(Ladds 2009)
primates, artiodactyls and pinnipeds	C	n.s.	n.s.	(Griner 1977)
Quokka (*Setonix brachyurus*)	C [c]	*C. gattii* (2), n.s. (3)	granulomatous pneumonia, pulmonary bronchiolar cryptococcosis [d]	Australian Registry of Wildlife Health (ARWH) database 1999-2014
	n.s.	*C. gattii*	n.s.	(Krockenberger *et al.* 2005)
	C	n.s.	n.s.	(Vaughan *et al.* 2007)
Tammar wallaby (*Macropus eugenii*)	C	n.s.	n.s.	(Ladds 2009)
tree shrews (*Tupaia tana*, *Tupaia minor*) and elephant shrews (*Macroscelides proboscides*)	C	n.s.	n.s.	(Tell *et al.* 1997)
Short-beaked echidna (*Tachyglossus aculeatus*)	C	n.s.	n.s.	(Ladds 2009)
striped grass mouse (*Lemniscomys barbarus*)	C	n.s.	pulmonary disease	(Bauwens *et al.* 2004)
Australian king-parrot (*Alisterus scapulatus*)	C	n.s.	n.s.	(Payne *et al.* 2005)
Schmidt's red-tailed guenon (*Cercopithecus ascanius*)	C	n.s.	multifocal infection	(Nevitt *et al.* 2013)
Blackbuck (*Antilope cervicapra*), plains rat (*Pseudomys australis*), greater stick-nest rat (*Leporillus conditor*), eastern barred bandicoot (*Perameles gunnii*), spinifex hopping mouse (*Notomys alexis*), bilby (*Macrotis lagotis*), ringtail possum (*Pseudocheirus peregrinus*), and northern quoll (*Dasyurus hallucatus*)	C	*C. neoformans* (variety not reported), *C. neoformans* var. *neoformans*, *C. gattii*, *Cryptococcus* spp.	various forms of cryptococcosis including, cerebellar, intestinal, meningoencephalitic, multisystemic, nasal, and pulmonary	ARWH, database 1999-2014
Common pigeon (*Columba livia*)	FR	*C. magnus*	apparently healthy	(Danesi *et al.* 2014)
Great cormorant (*Phalacrocorax carbo*)	FR	*C. magnus*	apparently healthy	(Danesi *et al.* 2014)
Malleefowl (*Leipoa ocellata*)	C	*C. neoformans*	systemic	ARWH database 1999-2014

Wildlife host species	C/FR	Isolate	Observations	Reference
Pesquet's parrot (*Psittrichas fulgidus*)	C	n.s.	osteomyelitis	(Molter, Zuba, & Papendick 2014)

[a] greater prevalence/incidence of *C. gattii*, species also recognised as the only cause of clinical infection; [b] greater prevalence of *C. neoformans* than *C. gattii*; [c] five cases reported; [d] infection considered to be incidental - cryptococcosis considered secondary cause of disease; n.s.= not specified.

Of all five cases involving cryptococcosis in quokkas archived in the Australian Registry of Wildlife Health (ARWH) (Table 5-1), species status of the *Cryptococcus* detected was only achieved in only two cases (both *C. gattii*; no varietal ID). The presence of cryptococcal organisms was considered to be incidental and cryptococcosis in captive quokkas was considered secondary to other causes of disease. According to Vaughan et al. (2007), there appear to be cases of cryptococcosis (details not provided) in quokkas in the archives of the Department of Agriculture and Food of Western Australia-Animal Health Laboratory (DAFWA-AHL). Retrieval of specific information on the cases reported by Vaughan et al. (2007) by this project was not possible, as it was indicated that there were no cases of cryptococcosis in quokkas in the archives of DAFWA-AHL (Dr Nicky Buller, Senior Microbiologist and Group Leader for Bacteriology and Mycobacteriology *pers. comm.* 2013).

As part of a health and disease baseline assessment for free-ranging quokkas, we used cultural and molecular methods to study yeasts isolated from nasal swabs collected from n=130 free-ranging *S. brachyurus* on Rottnest Island and the mainland of WA.

5.3 Materials and Methods

Between September 2010 and December 2011, 153 quokkas were captured on Rottnest Island and on the mainland in southwest WA. For trapping site descriptions, general procedures including physical examination, and peripheral blood collection techniques, refer to Chapter 3, section 3.3. Thorough PEs were carried out on all animals. Animals (n= 130) were tested for *Cryptococcus* spp. (Rottnest Island= 97 and mainland= 33) by culturing methods. Extracted DNA from isolated suspicious colonies was amplified by PCR and subsequently characterised by sequencing.

Nasal swabs were collected in the field from each nostril of each animal by gently introducing a swab (Minitip Aluminum Wire Venturi Transystem®, Copan Italia S.p.A, Brescia, Italy) 1.5-2 cm into the nasal vestibule and rotating it at least 10 times against the nasal lining. Extreme care was put on not touching the skin surrounding the nares upon insertion of the swab. Any swab that contacted with any surface other than the nasal lining was discarded. Duplicate swabs were collected from 45 of the 130 animals sampled. Swabs were then placed into Amie's transport medium without charcoal and stored at 4 ˚C until processing. A subset of swabs was stored at -20˚C for later processing if necessary.

Blood samples were collected to enable comparison of HMT and BLC profiles with the presence of *Cryptococcus* spp. Blood from the lateral tail vein was collected into EDTA and lithium heparin, and then sent to the Clinical Pathology service of the Murdoch University Veterinary Hospital for HMT and BLC studies. In-house differential counts were done manually on blood smears. PBCM (red and white blood cells) were visually recorded from blood smears (e.g. keratocytes, schistocytes, reactive lymphocytes, and atypical neutrophils -including toxic changes-). Microfilariae were screened for from blood smears, while *Theileria* sp. and *Babesia* sp. were screened for by nPCR (see Chapter 7, section 7.3.1). Macropod herpesvirus 6 (MaHV-6) was screened for by nPCR (see Chapter 6, section 6.3.1), and nematode eggs and *Eimeria* sp. oocysts were screened from faeces by light microscopy (see Chapter 3, section 3.4.1). Lastly, *Salmonella* spp. were screened for by culture methods and identified by serotyping (see Chapter 4, section 4.2.1).

A number of parameters were measured in this study: white blood cell counts (corrected to exclude nucleated red blood cells) (WBC), red blood cell concentration (RBC), haemoglobin concentration (HGB), packed cell volume (PCV), the absolute concentrations for neutrophils (NEUT), eosinophils (EOS), basophils (BASO), lymphocytes (LYMPH) and monocytes (MONO). Blood chemistry analytes included: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), total protein (PROT), albumin (ALB), calcium (CA), phosphorus (P), cholesterol (CHOL), total bilirubin (BILT), glucose (GLUC), creatinine (CREAT), urea (UREA), and vitamin E (Vit. E).

5.3.1 ***Laboratory techniques for Cryptococcus spp.***

5.3.1.1 Isolation and storage

Isolation and manipulation of fungal cultures and all related procedures were performed in a class II laminar flow biosafety cabinet (LAF Technologies Pty Ltd., North Ringwood, Australia). Swabs were analysed for yeasts by various laboratories (Table 5-2).

Table 5-2 Processing of nasal swabs (n= 130) collected from quokkas (*Setonix brachyurus*) from Rottnest Island (RI) and the mainland (ML) of southwest Western Australia for isolation and analysis of yeasts.

Number of swabs	Year	Collection site	Analysed
8	2010	ML	Clinical Pathology, Murdoch University Vet. Hospital
15	2011	RI	Mycology Department, PathWest, Perth
12	2011	RI	VetPath, Perth
95	2011	ML and RI	In-house

Isolation of *C. neoformans* var. *grubii* and *C. magnus* was achieved by inoculating a swab onto two plates of bird seed agar (BSA) (Faculty of Veterinary Science, The University of Sydney). Bird seed agar is used as a medium that allows for the differentiation of *C. neoformans* (i.e. var. *grubii* and var. *neoformans*) and *C. gattii* colonies with brown colony effect (BCE), from colonies of non-*neoformans* non-*gattii Cryptococcus*, that do not produce BCE (Staib *et al.* 1987). Bird seed agar contained penicillin (20 units mL^{-1}) and gentamicin (80 mg mL^{-1}) to suppress the growth of bacteria.

All suspicious colonies in the first two BSA plates (i.e. BCE or yeast like growth) were sub-cultured again on fresh BSA plates, which were then subcultured onto two Sabouraud dextrose agar (SDA) plates (Microbiology, School of Veterinary & Biomedical Sciences, Murdoch University). One SDA plate was incubated at 37 ˚C in order to confirm growth at mammalian temperature, and the others at 28 ˚C. Plates were checked daily for growth and contamination. Preliminary confirmation of *Cryptococcus* spp. like-yeast organisms was done through a series of tests (i.e. Gram stain, India ink, Christensen's Urea Agar [CUAT]) on colonies from SDA plates incubated at 28 ˚C, (see Appendix 10.2.1). Colonies from SDA pure cultures were stored in Microbank™ System (PRO-LAB Diagnostics, Richmond Hill, Canada) cryovials according to the manufacturer's guidelines (see Appendix 10.2.1). Isolation and storage of other *Cryptococcus* spp. and other yeast isolates were identical.

5.3.1.2 Revival and preliminary identification

Initial identification of the isolated yeast-like organisms was achieved by biochemical means. For this, isolates in Microbank™ cryovials were revived by subculturing on SDA and incubating at 28 ˚C for 3-5 days before growth started to appear. Preliminary identification was carried out using the API® 20 C AUX identification system for yeast (bioMérieux SA, Marcy-I'Etoile, France). Test strips were incubated at 28 ˚C and readings carried out at 48 h and 72 h: a positive reading was a cupule with greater turbidity than the control cupule. Interpretation of the numerical profile obtained (according to the result of each cupule) was done using the apiweb™ identification software with database v.4.0. For the details of yeast revival and preliminary identification protocols see Appendix 10.2.2.

5.3.1.3 DNA extraction and molecular identification

We initially tested the performance of a modified protocol (see Appendix 10.2.3) of the PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, USA) against two known protocols (called control protocols) for yeast DNA extraction: DNAzol® Reagent (Invitrogen™, Carlsbad, USA) and the Gram-positive protocol of QIAamp® DNA Mini (QIAGEN®, Hilden, Germany), by extracting DNA from two fungal isolates using all three protocols. Purity and quantity of the extracted DNA products from the three different kits used were compared using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific™, Waltham, USA). The ratio of absorbance at 260 nm and 280 nm (260/280) as well as the concentration of DNA/RNA recovered per µL were similar for all three. Considering these results, DNA from all remaining isolates was extracted using the PowerSoil™ DNA isolation Kit from MO BIO Laboratories, Carlsbad, USA.

All isolates were characterised by polymerase chain reaction (PCR) (targeting the Internal Transcribed Spacer (ITS) regions in the ribosomal Deoxyribonucleic Acid (rDNA) and sequencing (see Appendix 10.2.3). The ITS region is widely accepted as the optimal target for identification of *Cryptococcus* spp., *Candida* spp., and *Trichosporon* spp. among other fungal organisms (Hsu *et al.* 2003 ; Petti 2007). Polymerase chain reaction products were visualised in 2% agarose gels made of 0.5x TBE buffer, stained with 1x SYBR-Safe (Life Technologies, Carlsbad, USA) and ran at 90 V, 400 mAmp for 45 min. DNA bands of the expected size were cut out using separate sterile scalpel blades and purified using the filter tip method (see Appendix 10.2.3). Purified PCR products were then sequenced in both directions using forward and reverse primers. Sequencing reactions were subsequently purified using the ethanol precipitation method (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Scoresby, Australia). Final sequencing was carried out on an ABI 3790 96 capillary automatic sequencer (Applied Biosystems, Scoresby, Australia). Sequences were analysed and cleaned using 4Peaks version 1.7.2 (A. Griekspoor and Tom Groothuis, at http://nucleobytes.com/4peaks/index.html).

Molecular identification of isolates to the species level was achieved by comparing predicted nucleotide sequences with sequences available in GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTN v.2.2.28+. For isolates of *C. neoformans* (confirmed by biochemical and molecular testing), identification to the variety level was achieved by sequence analysis of the ITS region, an economic and highly reproducible accepted target for barcoding of fungi (Katsu *et al.* 2004 ; Meyer *et al.* 2011). At least two sequences of each previously-identified major molecular types of the *Cryptococcus neoformans* complex (VNI, VNII, VNIIIA-serotype A allele of the AD hybrid, VNIV and

VNIIIB-serotype D allele of the AD hybrid) (Table 5-3) were downloaded from the DNA Data Bank of Japan [DDBJ] server (http://www.ddbj.nig.ac.jp/). Query sequences were then aligned to the DDBJ sequences with MUSCLE (Edgar 2004) using MEGA5 v. 5.2.2 (Tamura *et al.* 2011), and visually inspected to determine specific ITS signatures according to combinations of eight nucleotides at positions 10, 11, 15, 19 and 108 in the ITS1 region, position 221 in the 5.8S rDNA unit, and positions 298 and 346 in the ITS2 region (Katsu *et al.* 2004) (Table 5-3). These ITS signatures have already been established to correlate to accepted molecular types obtained by use of other techniques such as random amplification of polymorphic DNA (RAPD) and PCR fingerprinting, among others (Meyer *et al.* 2011).

Table 5-3 List of *C. neoformans* reference ITS nucleotide signatures 5'-3' used in this study, with their corresponding serotype, PCR fingerprint type and ITS type.

DDBJ accession number	Origin	Serotype	PCR fingerprint type	ITS type	Nucleotide signature 5'-3'
AJ493550	Human, CSF, USA	A	VNI	1	ATACTAGC
AJ493551	Human, CSF, Australia	A	VNI	1	ATACTAGC
AJ493552	Human, CSF, Australia	A	VNII	1	ATACTAGC
AJ493554	Wood, Zaire	A	VNII	1	ATACTAGC
AJ493559	Human, CSF, USA	D	VNIV	2	ATATAGGC
AJ493561	Human, blood, Australia	D	VNIV	2	ATATAGGC
AJ493555	Human, Germany	AD	VNIII	2	ATATAGGC
AJ493558	Human, CSF, Australia	AD	VNIII	2	ATATAGGC

CSF= cerebrospinal fluid

5.3.2 *Statistical analyses*

Haematology, BLC, and PBCM datasets for animals that tested positive or negative to *Cryptococcus* spp., were analysed independently due to differences in sample sizes between these datasets (some individuals were tested for either HMT or BLC, and not all individuals had a blood smear, which would mean their exclusion from analyses of the entire dataset due to missing data). *Sex* was used as a covariate in all three analyses. Data were explored visually with non-metric Multi-Dimensional Scaling (nMDS) and a Bray-Curtis similarity measure (Bray & Curtis 1957) using PAST v. 3.02 (Hammer, Harper, & Ryan 2001). Haematology and BLC response variables were fitted to an approximate normal distribution (BoxCox transformation; STATISTICA v. 9.1, StatSoft Inc.). Due to insufficient observations as well as limitations of the multivariate analysis tests used, seasonal differences were explored using χ^2 for Trend.

Haematology and BLC dependant variables were range-standardised to a scale between 0 and 1, while PBCM were not as it was a binary dataset. For each nMDS plot, two-or-three-dimensional analyses were selected according to the model that had the lowest stress statistic to determine adequacy of the fit. To determine the similarity or dissimilarity of

the HMT, BLC, and PBCM datasets between the groups of interest (*Cryptococcus*-positive and -negative) a pairwise Similarity Percentage (SIMPER, PAST v. 3.02) analysis (Clarke 1993) using Bray-Curtis similarity measure (Bray & Curtis 1957) was carried out. SIMPER results (i.e. percent of contribution of each variable to the similarity or dissimilarity) are accompanied by the arithmetic mean ($\bar{x}$) and standard deviation (SD) for each HMT and BLC variable, while odds ratio (OR) and 95% confidence intervals (CI) were calculated (Woolf 1955) for each PBCM observed in blood smears. The significance of the observed differences in the HMT, BLC and PBCM profiles between *Cryptococcus*-positive and -negative animals, was examined using a two-way non-parametric permutational multivariate analysis of variance (Two-way PERMANOVA, PAST v. 3.02) (Anderson 2001) with 9,999 permutations. This test provides a *Pseudo-F* statistic that is used to compare variability between groups from variability within groups, in which the larger the value of *F*, the more likely is that the null hypothesis of no variability between groups is false (Anderson 2001). A *p*-value is then use to detect the significance of each factor in the design and the interaction between them.

Associations between *Cryptococcus* spp. with sex, physical examination findings and concomitant infection with *Babesia* sp., *Eimeria* sp., macropodid herpesvirus 6 (MaHV-6), microfilariae, nematode eggs, *Theileria* sp., and *Salmonella* spp., were explored using χ^2 test with Yates' correction. OR and 95% CI were calculated using the Woolf's method (1955). When a null value was present in a contingency table, 0.5 were added to each observed value in order to calculate OR and 95% CI (Altman 1999). All other CI for estimates of proportions (i.e. prevalence), were calculated using the Wilson model for $n \leq 40$, and the Jeffreys model for $n \geq 40$ (Brown, Cai, & DasGupta 2001). Significance was set at $p < 0.05$ for all analyses, unless stated differently.

For the presence or absence of potential plant and animal sources of *C. neoformans* var. *grubii* in the natural habitat of *S. brachyurus*, we used 'FloraBase v. 2.8.17', an online resource for Western Australian flora (http://florabase.dpaw.wa.gov.au/) (Western Australian Herbarium 1998), NatureMap v. 1.7.0.15 a mapping interface for Western Australia's biodiversity, an initiative of the Department of Parks and Wildlife (DPaW) and the Western Australian Museum (http://naturemap.dpaw.wa.gov.au/) (Department of Parks and Wildlife (DPaW) 2007); the Atlas of Living Australia an initiative of the Australian Government (http://www.ala.org.au), and the Australian Plant Name Index (APNI), a tool of the Integrated Biodiversity Information System (IBIS), the online database of the Australian National Herbarium and the Australian National Botanic Gardens (http://www.anbg.gov.au/apni/index.html).

5.4 Results

5.4.1 *Phenotypic characteristics and molecular identification*

Molecular analyses (below) enabled retrospective identification of the colony properties of each *Cryptococcus* isolate:

***C. neoformans* var. *grubii* (RIA0075 and RIA0082)** isolates were identified as *C. neoformans* by the API system, with an identification score of 99.9% (API numerical profile 2557373). On BSA at 28 ˚C, all isolates produced colonies with BCE (Figure 5-1a) that were mostly circular, smooth, somewhat effuse, opaque and mucoid. Microscopically, yeast cells were generally budding, with circular appearance (Figure 5-1c). Cells presented a mucopolysaccharide capsule under India ink (Figure 5-1d). By API® 20 C AUX strip analysis, isolates assimilate glucose, calcium 2-keto-gluconate, D-xylose, adonitol, D-galactose, inositol, D-sorbitol, Methyl-α D-glucopyranoside, N-acetyl-glucosamine, D-cellobiose, D-maltose, D-saccharose (sucrose), D-trehalose, D-melezitose and D-raffinose. Isolates did not assimilate D-lactose, xylitol, L-arabinose and glycerol.

***C. magnus* (isolate AS3413)** isolates were assigned API numerical profile 2705373 for which there is no identification in the apiweb™ identification software (database v.4.0). On BSA, colonies did not grow at 37 ˚C; at 28 ˚C they were light white-coloured and smaller than those of *C. neoformans* var. *grubii* at the same inspection time. Microscopically, yeast cells were generally budding, with ellipsoidal appearance (Figure 5-1f). Cells presented a mucopolysaccharide capsule under India ink (Figure 5-1g). Isolates were able to assimilate glucose, calcium 2-keto-gluconate, L-arabinose, D-xylose, inositol, Methyl-α D-glucopyranoside, N-acetyl-glucosamine, D-cellobiose, D-maltose, D-saccharose (sucrose), D-trehalose, D-melezitose and D-raffinose. Isolates did not assimilate glycerol, adonitol, xylitol, D-galactose, D-sorbitol, and D-lactose.

On SDA at 37 ˚C colonies of *C. neoformans* var. *grubii* grew avidly (<24 h) while colonies of *C. magnus* did not grow. At 28 ˚C colonies of both yeast were undistinguishable (Figure 5-1b,e), being dome-shaped, cream-coloured, circular, opaque and mucoid but not presenting BCE. Isolates of both *C. neoformans* var. *grubii* and *C. magnus* exhibited rapid hydrolysis of urea (<24 h) when inoculated on Christensen's Urea Agar.

Sequence analysis of the ITS regions for isolates previously identified (culture, biochemical and alignment with BLASTN) as members of the *C. neoformans* complex,

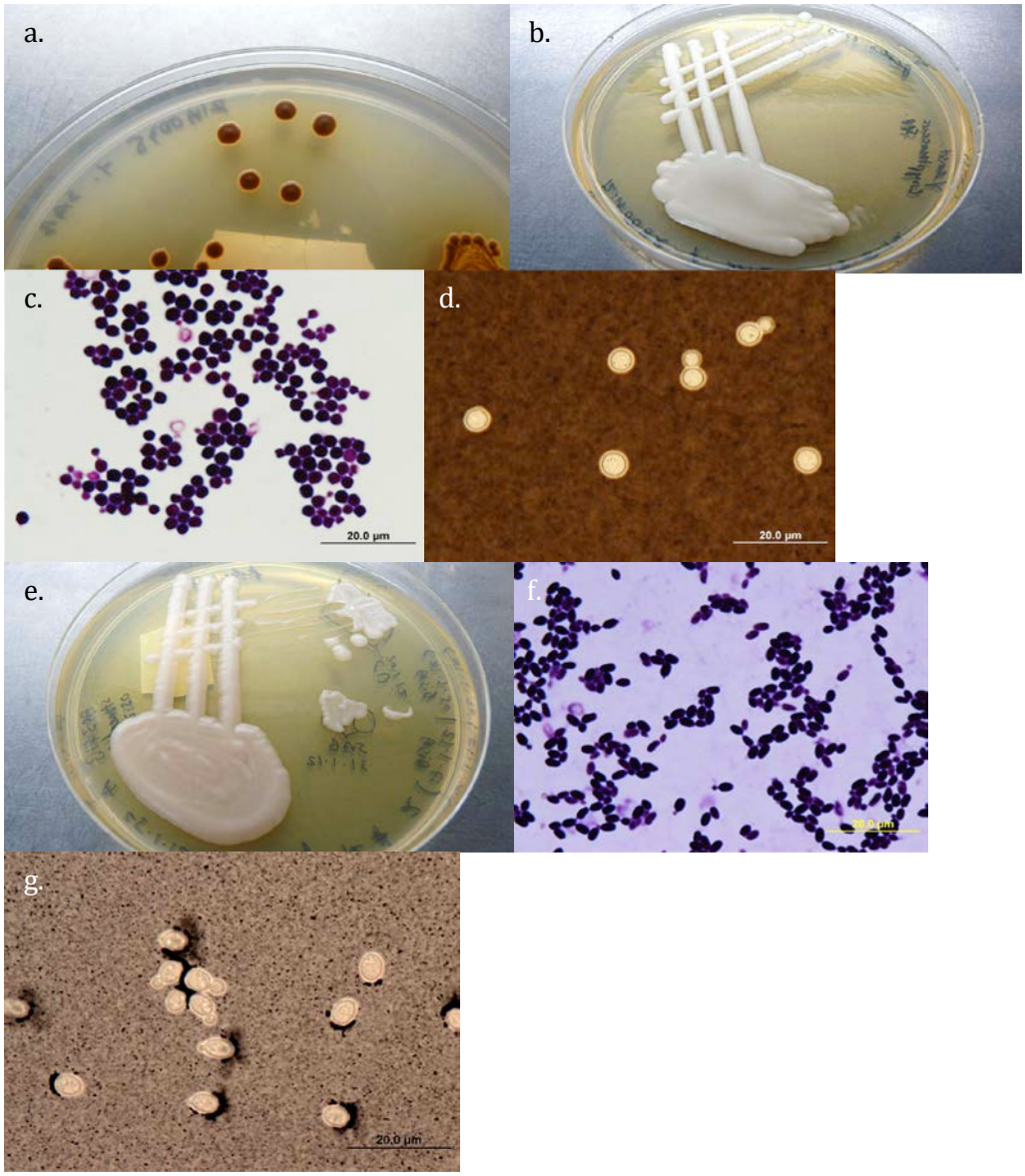


Figure 5-1 *C. neoformans* var. *grubii*: (a) BCE on BSA, (b) growth on SDA, (c) circular cells with budding on Gram stain, (d) spherical capsulated cells on India ink. *C. magnus*: (e) growth on SDA, (f) ellipsoidal cells with budding on Gram stain, (g) ellipsoidal capsulated cells on India ink.

indicated that isolates RIA0075 and RIA0082 both recovered from animals in the Settlement area, had identical ITS nucleotide signatures, with 5'-ATACTAGC-3' nucleotide combination at positions 10, 11, 15, 19 and 108 in the ITS1 region, position 221 in the 5.8S rDNA gene, and positions 298 and 346 in the ITS2 region (Katsu *et al.* 2004) (Figure 5-2), identifying the isolates as ITS type 1 (Table 5-3) or *C. neoformans* var. *grubii*. At the time of the analysis, isolate AS3413 was identified as *C. magnus*, according to BLASTN v.2.2.28+, with 99% identity with *C. magnus* Accession no. AB727344 (GenBank) (Figure 5-3). BLASTN v.2.2.28+ results for non-cryptococcal organisms recovered were *Aureobasidium pullulans, Rhodosporidium kratochvilovae*, *Rhodotorula glutinis*, and *Rhodotorula mucilaginosa.*

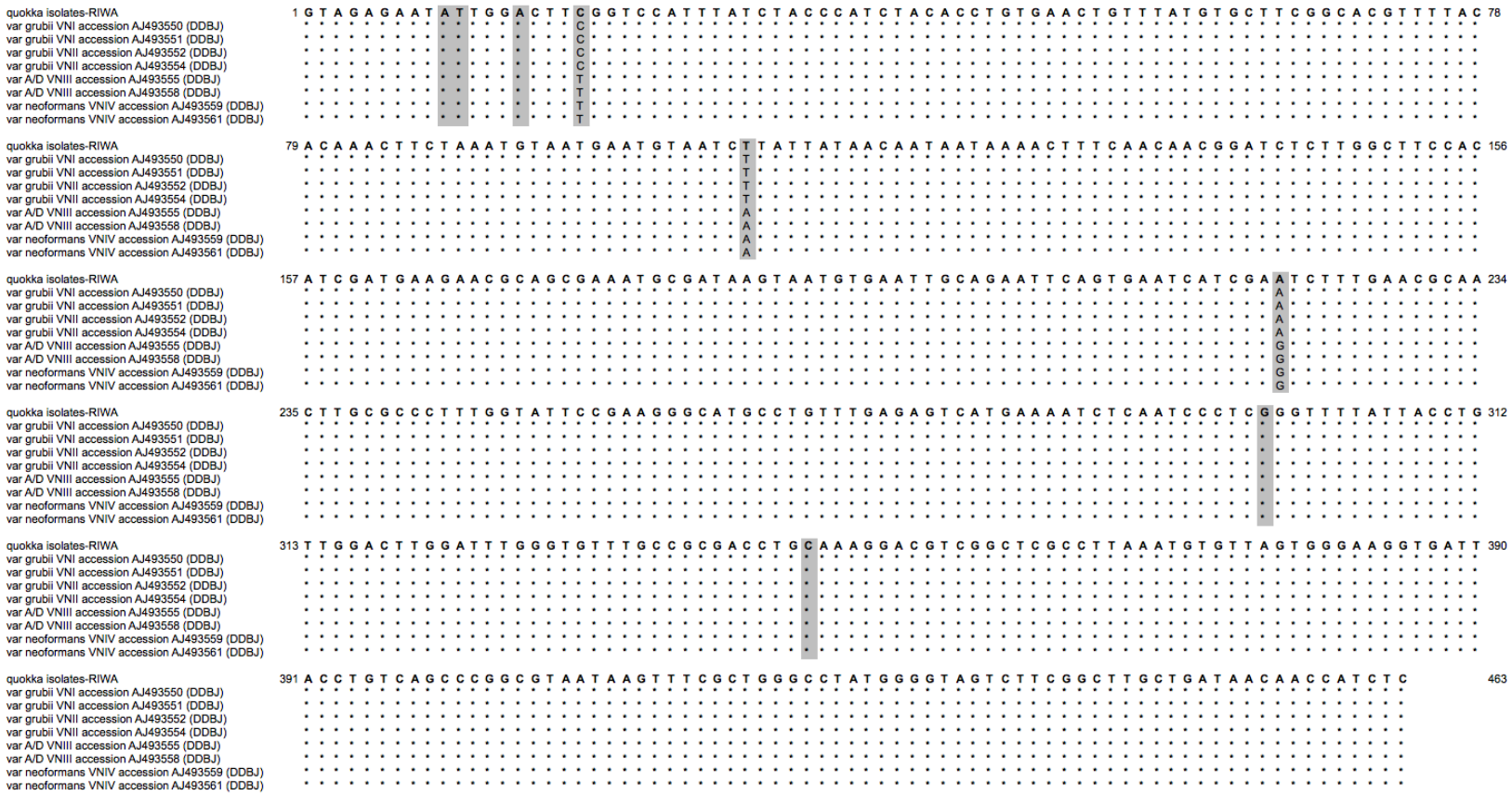


Figure 5-2 Muscle alignment of the ITS (ITS1-5.8s rRNA-ITS2) sequences of the *C. neoformans* complex and the various molecular types (VNI, VNII, VNII and VNIV), used to determine the serotype A of the isolates recovered from *S. brachyurus* on Rottnest Island (quokka isolates-RIWA -for Rottnest Island Western Australia-). Note the nucleotide 5'- ATACTAGC-3' signature at positions 10, 11, 15, 19, 108, 221, 298 and 346, indicated by grey columns.

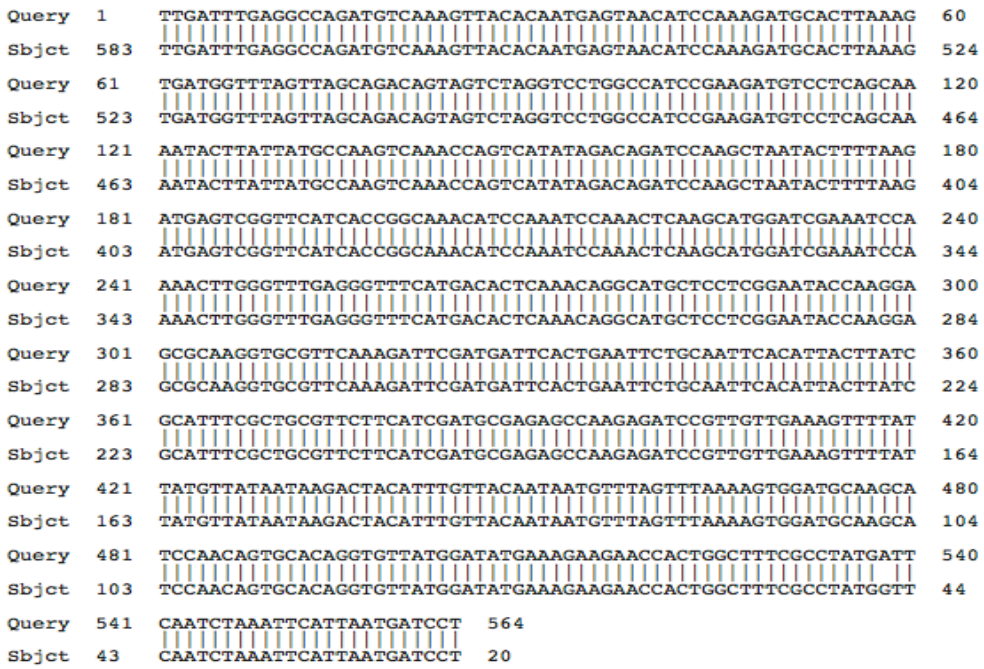


Figure 5-3 BLASTn v.2.2.28+ alignment of a 564 nucleotide segment (without primer sequences) of the ITS region (ITS1-5.8s rRNA-ITS2) of the fungal genome detected in *S. brachyurus* (= Query), aligned with the equivalent region of *C. magnus* (Accession no. AB727344 (= Sbjct) on GenBank.

5.4.2 *Distribution and prevalence of* all yeasts *recovered from the nasal lining of S. brachyurus (Rottnest Island and mainland)*

Cryptococcus spp. was isolated from 7 of 97 Rottnest Island animals screened (49 females and 48 males) for an estimated prevalence of 7.2% (CI 3.3-13.6). Unspeciated *Cryptococcus* spp. was recovered from four of these animals (Serpentine= 2, Barker Swamp= 2, 4.12%, CI 1.4-9.5), *Cryptococcus magnus* was recovered from an individual (1.03%, CI 0.1-4.7) captured at Kingston site, while *Cryptococcus neoformans* var. *grubii*, was recovered from two individuals (one male, one female) captured on the Settlement (2.1%, CI 0.4-6.4). Figure 5-4 illustrates the geographical distribution of the cryptococcal isolates. In total, 21 colonies of *C. neoformans* var. *grubii* were recovered from both animals (male= 7, female= 14). Other yeast and yeast-like fungi isolated were: *Rhodotorula glutinis* (1/97, 1.03%, CI 0.2-5.6), *Rhodotorula mucilaginos*a (2/97, 2.1%, CI 0.6-7.2), and *Aureobasidium pullulans* (5/97, 5.15%, CI 2.2-11) (Figure 5-5).

Cryptococcus spp. was not recovered from any of the 33 mainland animals (4 female and 29 male) that were screened (all subpopulations). Other yeasts recovered from the nasal lining of mainland quokkas were: *Rhodosporidium kratochvilovae* (4/33, 12.12%, CI 4.8-27), *Rhodotorula mucilaginosa* (2/33, 6.06%, CI 1.7-19.6), and *Rhodotorula glutinis* (2/33, 6.06%, CI 1.7-19.6).

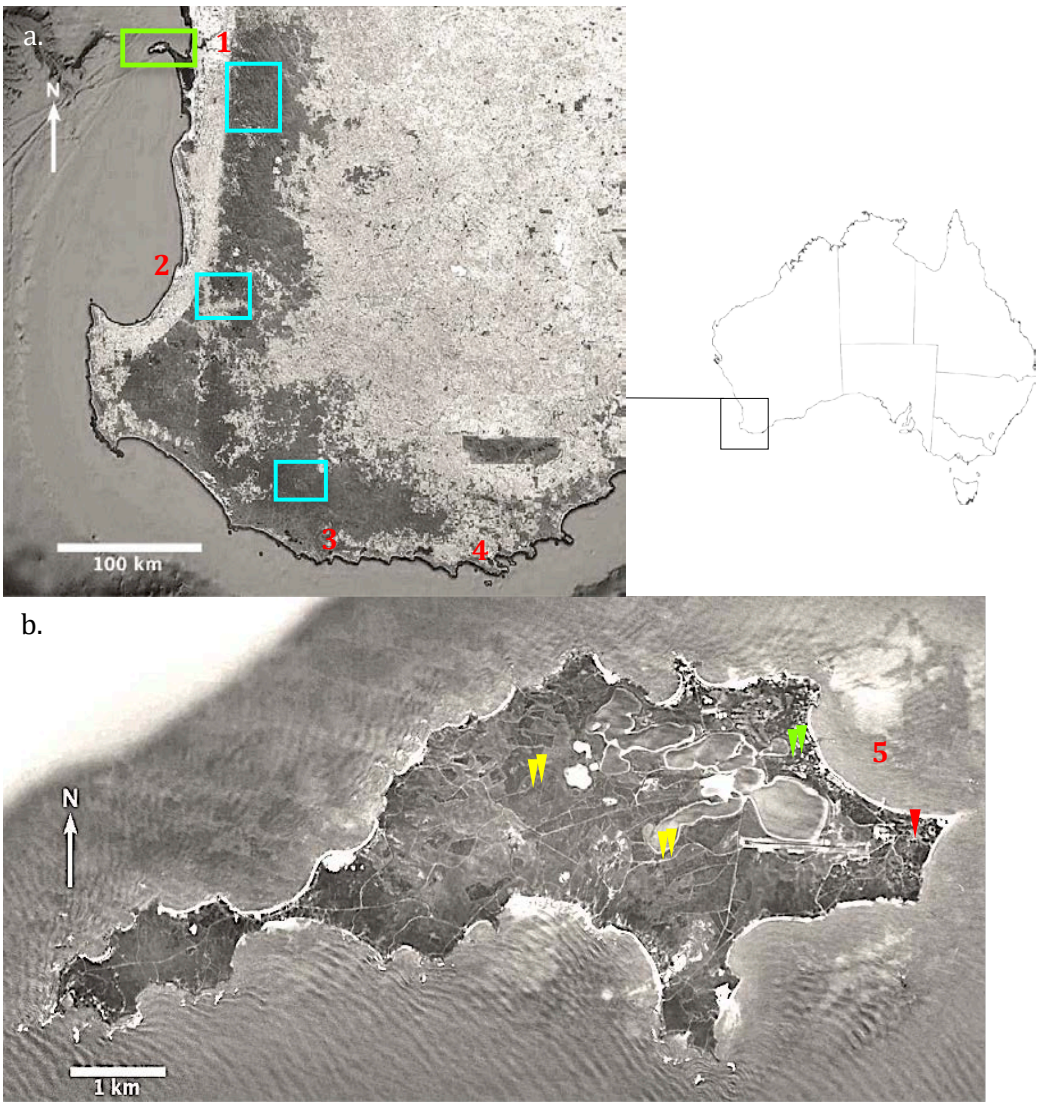


Figure 5-4 Geographic distribution of (a) major areas were animals were sampled (blue boxes= areas on the mainland sampled but were negative); numbers represent cities and towns: 1= Perth, 2= Bunbury, 3= Walpole, 4= Albany; and (b) spatial distribution of cryptococcal isolates recovered from quokkas on Rottnest Island (green box in figure a.), 5= Thompson's Bay. Each arrowhead represents a positive animal. Yellow arrowheads (*Cryptococcus* spp. left: Barker Swamp and right: Serpentine sites), green arrowheads (*C. neoformans* var. *grubii* - Settlement site), and red arrowhead (*C. magnus* - Kingston site). Map data ©2016 Google Landsat, Data SIO, NOOA, U.S. Navy, NGA, GEBCO.

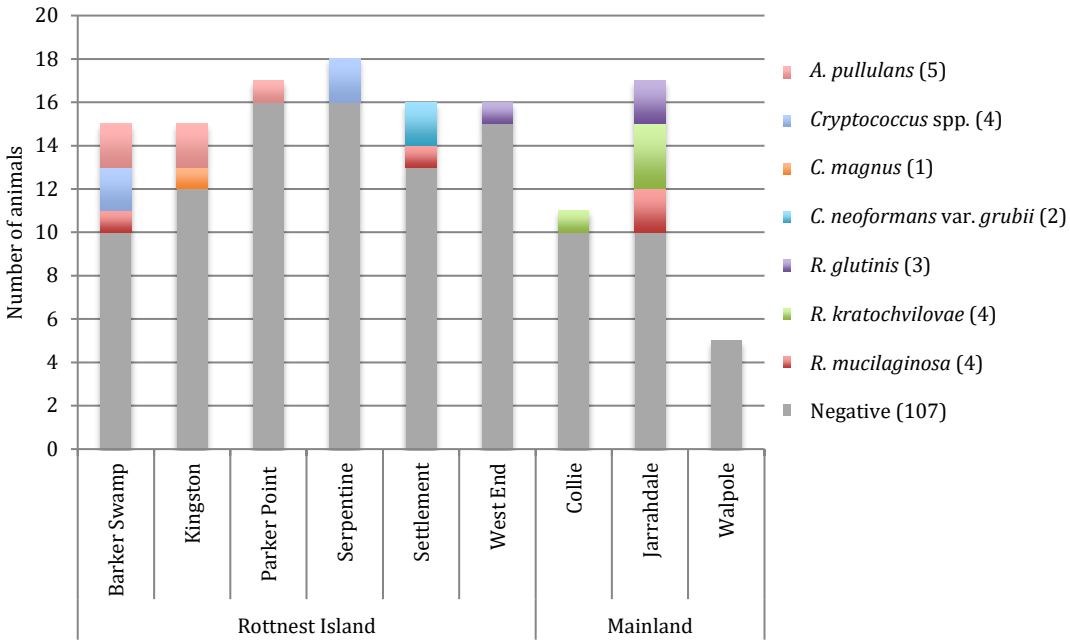


Figure 5-5 Nasal swabs tested for *Cryptococcus* spp. and other yeast and yeast-like fungi from *S. brachyurus* on six sites on Rottnest Island and three sites on the mainland. The number of isolations is given in parentheses.

The probability (i.e. OR) of females to be positive to *Cryptococcus* spp. was 6.56 (CI 0.76-56.7) times higher than males. However, this association was not significant [females (6/49 = 12.2%), males (1/48 = 2.1%); χ^2_1= 2.38, p= 0.123]. Similarly, there was no significant difference in the prevalence of *Cryptococcus* spp. in quokkas on Rottnest Island by season (χ^2_1= 2.98, p= 0.08; Figure 5-6).

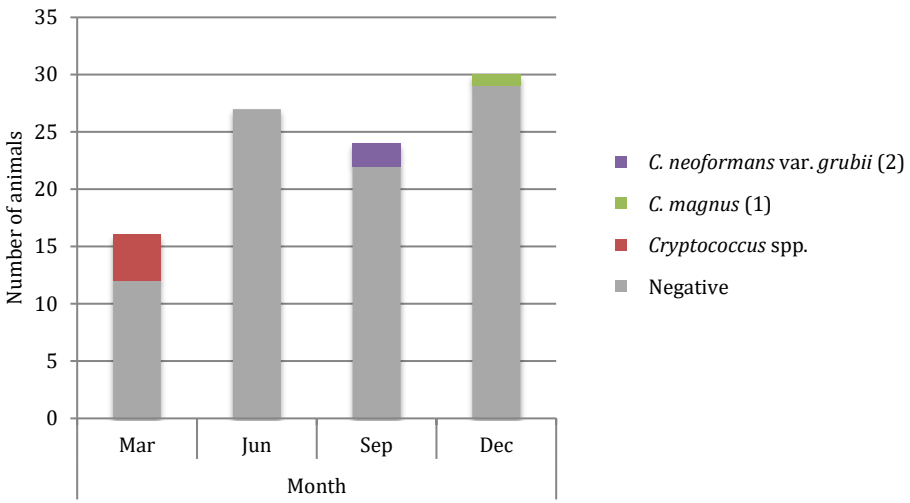


Figure 5-6 Animals positive and negative to *Cryptococcus* spp. on Rottnest Island between March and December 2011.

5.4.3 *Correlates of Cryptococcus spp. with HMT, BLC and PBCM variables for Rottnest Island S. brachyurus*

Subtle differences in the HMT, BLC and PBCM datasets between *Cryptococcus* spp. positive and negative animals were present (SIMPER; Table 5-4a,b,c). This was evident in the WBC (less NEUT, EOS, MONO and BASO, and higher LYMPH), erythrogram (lower RBC and PCV, and higher HGB), and BLC (lower PHOS, CK, UREA, and ALP; higher Vit. E, CHOL, BILT, and PROT) profiles of *Cryptococcus* spp. positive animals. However, these differences were not significant (PERMANOVA: $p < 0.05$, Table 5-5a,b,c). By contrast, there was a significant association between *sex* and differences in BLC profiles (PERMANOVA: $p < 0.05$; Table 5-5b) of quokkas on Rottnest Island. Both factors (i.e. *Cryptococcus* spp. and sex) appeared to have no significant interaction (PERMANOVA: $p < 0.05$; Table 5-5a,b,c).

Table 5-4 SIMPER analysis results indicating the contribution of specific variables to the observed differences in HMT (a), BLC (b), and PBCM (c) profiles of quokkas that were *Cryptococcus* spp.-positive and *Cryptococcus* spp.-negative on Rottnest Island. HMT= haematology, BLC= blood chemistry, PBCM= peripheral blood cell morphology.

		Taxon	Ct %	*Cryptococcus* spp. +ve		*Cryptococcus* spp. -ve	
				$\bar{x}$	SD	$\bar{x}$	SD
a.	HMT (23.5) † n +ve= 7 n -ve= 75	Lymphocytes (x10e9/L)	14.4	2.08	1.00	2.03	1.11
		Monocytes (x10e9/L)	13.1	0.05	0.03	0.07	0.06
		WBC (x10e9/L)	12.5	4.56	1.27	4.75	1.63
		Basophils (x10e9/L)	11.6	0.01	0.02	0.02	0.03
		RBC (x10e12/L)	11.2	5.81	0.50	5.87	0.93
		HGB (g/L)	10.4	110	8.87	109	15.4
		Eosinophils (x10e9/L)	9.58	0.32	0.18	0.38	0.31
		Neutrophils (x10e9/L)	8.89	2.11	0.39	2.25	1.00
		PCV (%)	8.28	33.3	2.69	33.6	4.48
b.	BLC (20.2) † n +ve= 7 n -ve = 84	Vit. E (mg/L)	10.8	7.59	1.69	6.31	1.72
		CHOL (mmol/L)	8.84	3	0.65	2.78	0.53
		PHOS (mmol/L)	8.13	1.1	0.50	1.22	0.47
		CK (U/L)	7.94	450	325	939	1,425
		UREA (mmol/L)	7.31	6.47	1.24	7.04	1.47
		BILT (µmol/L)	6.79	4.56	1.61	4.20	1.61
		ALP (U/L)	6.78	5,709	3,106	8,812	11,125
		GLUC (mmol/L)	6.77	4.84	1.40	4.11	2.31
		PROT (g/L)	6.72	61.7	2.57	60.4	4.76
		CALC (mmol/L)	6.71	2.19	0.17	2.20	0.20
		CREAT (µmol/L)	6.36	64.1	20.7	70.0	14.9
		ALT (U/L)	6.25	214	37.8	224	65.9
		ALB (g/L)	5.90	36.4	1.25	36.5	1.81
		AST (U/L)	4.73	44.3	13.1	49.5	32.0

				Cryptococcus spp. +ve	*Cryptococcus* spp. -ve	
			Ct %	Frequency (%)	Frequency (%)	OR ‡ (95% CI)
c.	PBCM (28.4) † +ve= 7 -ve= 83	Rouleaux formation	12.0	4 (57)	35 (42)	1.83 (0.38-8.69)
		Acanthocytes	11.6	2 (29)	42 (51)	0.39 (0.07-2.13)
		Heinz bodies	11	1 (14)	39 (47)	0.19 (0.02-1.63)
		Poikilocytosis	10.6	4 (57)	75 (90)	0.14 (0.03-0.75)
		Echinocytes	9.30	2 (29)	20 (24)	1.26 (0.23-7.00)
		Hypochromasia	7.83	5 (71)	76 (92)	0.23 (0.04-1.41)
		Anisocytosis	7.56	6 (86)	65 (78)	1.66 (0.19-14.7)
		Flower Cells	6.95	1 (14)	19 (23)	0.56 (0.06-4.96)
		Keratocytes	6.50	1 (14)	17 (20)	0.65 (0.07-5.74)
		Schistocytes	5.88	1 (14)	14 (17)	1.37 (0.34-5.43)
		Howell-Jolly bodies	5.01	6 (86)	77 (93)	0.47 (0.05-4.54)
		nRBCs	2.78	7 (100)	74 (89.2)	1.91 (0.10-36.2) *
		Polychromasia	2.64	7 (100)	75 (90)	1.69 (0.09-32.3) *
		Reactive lymphocytes	0.40	7 (100)	82 (99)	0.27 (0.01-7.30) *

Ct: Percent of contribution to difference
† Overall average dissimilarity
‡ Odds ratio for the presence of the taxon in *Salmonella* +ve individuals and 95% confidence intervals calculated using Woolf's method (1955)
* calculated by adding 0.5 to each observed value (Altman 1999)

Table 5-5 Two-way PERMANOVA of selected HMT variables (a) (corrected WBC, RBC, HGB, PCV, and absolute counts for leukocytes obtained with a manual differential on a blood smear), BLC analytes (b) (ALP, ALT, AST, CK, PROT, ALB, CALC, PHOSP, CHOL, BILT, GLUC, CREAT, UREA and Vitamin E), and PBCM (c) for *Cryptococcus* spp.-positive and *Cryptococcus* spp.-negative *S. brachyurus* on Rottnest Island, with sex as independent factor. Bray-Curtis similarity index, Permutation N= 9,999. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

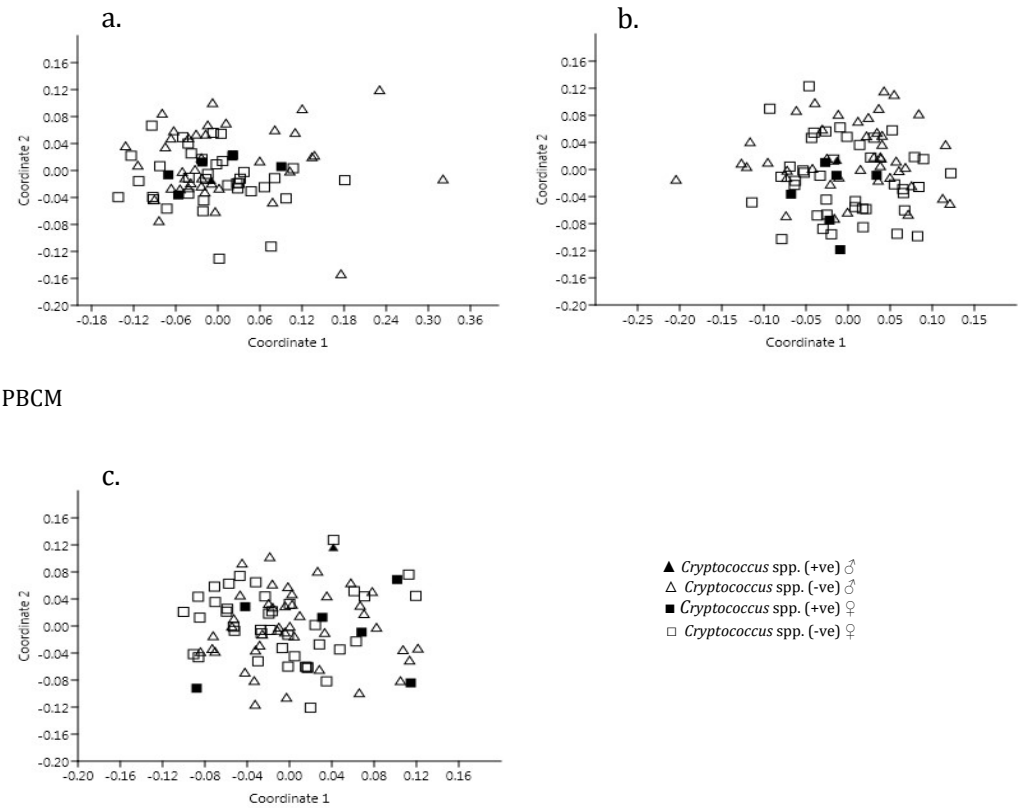
	Data	Factor	SS	df	MS	*F*	*p*
a.	HMT	*Cryptococcus* spp.	0.014	1	0.014	0.179	0.897
		Sex	0.027	1	0.027	0.342	0.704
		Interaction	-2.81	1	-2.81	-35.2	0.967
		Residual	6.21	78	0.080		
		Total	3.45	81			
b.	BLC	*Cryptococcus* spp.	0.020	1	0.020	0.462	0.583
		Sex	0.053	1	0.053	1.220	**0.024**
		Interaction	-1.71	1	-1.71	-39.4	0.955
		Residual	3.77	87	0.043		
		Total	2.13	90			
c.	PBCM	*Cryptococcus* spp.	0.055	1	0.06	0.862	0.216
		Sex	0.022	1	0.02	0.347	0.652
		Interaction	-2.37	1	-2.37	-37.1	0.312
		Residual	5.50	86	0.06		
		Total	3.21	89			

SS: sum of squares
df: degrees of freedom
MS: mean sum of squares

There was no particular clustering of the HMT, BLC and PBCM data either across *Cryptococcus* spp. status (i.e. -positive, -negative) or across sexes (nMDS analyses: Figure 5-7a,b,c).

HMT

BLC



PBCM

Figure 5-7 Non-metric MDS plots with Bray-Curtis similarity measure, illustrating the structural dissimilarity of HMT (a. stress statistic= 0.177), BLC (b. stress statistic= 0.271) and PBCM (c. stress statistic= 0.28) communities in *Cryptococcus* spp.-positive and *Cryptococcus* spp.-negative *S. brachyurus* from Rottnest Island. Key legend applies for all plots. Note that the distances along the axes are unit-less, therefore the positions of the points in the plots are relative distances from one another rather than absolute differences read in these units. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

5.4.4 *Relation between Cryptococcus spp. and: (i) physical examination findings, (ii) other organisms*

There were no differences in the physical examination findings between animals positive or negative for *Cryptococcus* spp. (Table 5-6). Similarly, there were no significant associations between the presence of other concomitant organisms (i.e. MaHV-6, piroplasms, *Salmonella*, microfilariae, nematode eggs, and *Eimeria* spp. oocysts) with the presence of *Cryptococcus* spp. in the nasal lining of the quokkas on Rottnest Island (Table 5-7).

Table 5-6 Results for associations between physical examination findings and saprophyte fungi in animals from Rottnest Island and the mainland (combined sample). OR= odds ratio, CI= confidence interval, CRT= capillary refill time, MM= mucous membranes, EP= external parasites.

	Cryptococcus spp.			
Finding	+ve (%) $_{n=7}$	-ve (%) $_{n=90}$	OR (95% CI)	$\chi^2_{1, n=97}$
Abnormal mentation [a]	1 (1.03)	8 (8.25)	1.71 (0.18-16)	= 0.05, p= 0.838
Abnormal CRT [b]	0 (0)	8 (8.89)	0.65 (0.03-12.3) *	= 0.01, p= 0.912
Abnormal MM [c]	1 (14.3)	27 (30)	0.39 (0.04-3.39)	= 0.20, p= 0.652
Cloudy eye	0 (0)	2 (2.22)	2.36 (0.10-53.8) *	= 0.96, p= 0.326
Dehydration [d]	2 (28.6)	46 (51.1)	0.38 (0.07-2.08)	= 0.57, p= 0.449
Ear notches	1 (14.3)	13 (14.4)	0.99 (0.11-8.88)	= 0.29, p= 0.584
Flaky skin	0 (0)	2 (2.22)	2.36 (0.10-53.8) *	= 0.96, p= 0.326
Fur loss	1 (14.3)	22 (24.4)	0.52 (0.06-4.52)	= 0.02, p= 0.882
Presence of EP	0 (0)	10 (11.1)	0.51 (0.03-9.61) *	= 0.08, p= 0.774
Skin erosions	0 (0)	2 (2.22)	2.36 (0.10-53.8) *	= 0.96, p= 0.326

[a] Diminished response to external stimuli
[b] Greater than two seconds
[c] Pale and blue tinted mucous membranes
[d] Skin tenting for longer than 2 seconds
[e] Ulcers found inside the pouch of females
* calculated by adding 0.5 to each observed value (Altman 1999)

Table 5-7 Results for associations of other infectious agents occurring concomitantly in animals from Rottnest Island tested for *Cryptococcus* spp. OR= odds ratio, CI= confidence interval, MaHV-6= Macropodid herpesvirus 6.

	Cryptococcus spp.			
Finding	+ve/n (%)	-ve/n (%)	OR (95% CI)	χ^2
Babesia sp. [a]	0/7 (0)	1/72 (1.39)	3.18 (0.12-85.1) *	$\chi^2_{1, n=79}$ = 2.12, p= 0.145
Eimeria spp. [b]	3/3 (100)	42/52 (80.8)	1.73 (0.08-36.1) *	$\chi^2_{1, n=52}$ = 0.01, p= 0.944
MaHV-6 [a]	0/7 (0)	6/72 (8.33)	0.68 (0.04-13.3) *	$\chi^2_{1, n=79}$ = 0.01, p= 0.962
Microfilariae [b]	3/7 (42.9)	28/85 (32.9)	1.53 (0.32-7.29)	$\chi^2_{1, n=92}$ = 0.01, p= 0.906
Nematode eggs [b]	3/3 (100)	46/51 (90.2)	0.83 (0.04-18.2) *	$\chi^2_{1, n=54}$ = 0.21, p= 0.648
Salmonella spp. [c]	1/3 (33.3)	25/56 (44.6)	0.62 (0.05-7.24)	$\chi^2_{1, n=59}$ = 0.04, p= 0.831
Theileria sp. [a]	2/7 (28.6)	16/72 (22.2)	1.40 (0.25-7.91)	$\chi^2_{1, n=79}$ = 0.01, p= 0.928

[a] Screened by PCR [MaHV-6: see Chapter 6, section 6.3.1; Piroplasms: Chapter 7, section 7.3.1.1]
[b] Screened by light microscopy [gastrointestinal parasites: see Chapter 3, section 3.4.1; microfilariae: see Chapter 7, section 7.3.1]
[c] Screened by culture methods, species and serovars determined by serotyping (see Chapter 4, section 4.2.1)
* calculated by adding 0.5 to each observed value (Altman 1999)

5.5 Discussion

Cryptococcus neoformans var. *grubii* (serotype A) (prevalence= 2.1%, CI 0.4-6.4) and *C. magnus* (prevalence= 1.03%, CI 0.1-4.7), were recovered from nasal swabs (by microbiological methods) from free-ranging *S. brachyurus* from Rottnest Island and the southwest of WA. This constitutes the first report of serotype A and *C. magnus* on Rottnest Island and probably within the offshore territory of WA. Four more cryptococcal organisms (samples sent to a commercial laboratory) reported simply as '*Cryptococcus* spp. not *neoformans* not *gattii*' were recovered but not characterised (4.12%). The overall prevalence of cryptococcal organisms on Rottnest Island was 7.2% (7/97). Even though the probability (i.e. OR) of infection with *Cryptococcus* spp. was 6.56 (CI 0.76-56.7) times

higher for females than males, this was not statistically significant (p= 0.123). There was also no influence of season on the prevalence of cryptococcal organisms (p= 0.08) on Rottnest Island. Cryptococcal organisms were not recovered from quokkas on the mainland. Other fungal organisms recovered from quokkas on Rottnest Island and the mainland were *Rhodotorula glutinis*, *R. mucilaginos*a, *Aureobasidium pullulans*, and *Rhodosporidium kratochvilovae* spp. Multivariate analyses showed that *Cryptococcus* spp. had no effect on the HMT, BLC or PBCM profiles of infected animals. Similarly, there were no significant associations between physical examination findings and concomitant infections with the presence of *Cryptococcus* spp.

Previous studies have indicated that in Australia, cryptococcal isolates recovered from populated urbanised areas are typically *C. neoformans* var. *grubii* (serotype A) were as the isolates recovered from rural areas are typically *C. gattii* (Chen *et al.* 2000 ; Lester *et al.* 2004). On the mainland, *S. brachyurus* is generally found in bushlands with practically no human development, whereas on Rottnest Island the species lives in contact with man. This contact occurs to various degrees; unsettled areas like Barker Swamp, West End and Serpentine where there is little to no contact, and the Settlement and Parker Point (urbanised areas), where there is close contact between *S. brachyurus* and people (refer to Chapter 3, section 3.1.1). In this context, the isolation of *C. neoformans* var. *grubii* from animals on the Settlement and not from other trapping sites on Rottnest Island is not just in line with previous reported distributions of the organism (mainly at urbanised and populated areas), but these isolations are significant for public health reasons, including that Rottnest Island is a popular tourist attraction, with ~500,000 visitors a year (2014 data), that *C. neoformans* var. *grubii* strains are generally more virulent than *C. neoformans* var. *neoformans* strains. Furthermore, *C. neoformans* var. *grubii* is responsible for the majority of cryptococcal infections in immunocompromised humans and animals (Janbon *et al.* 2014 ; Nielsen *et al.* 2003 ; Steenbergen & Casadevall 2000 ; Sykes & Malik 2012).

The overall prevalence of cryptococcal species on Rottnest Island (*Cryptococcus* spp., prevalence= 4.12%; *C. neoformans* var. *grubii*, prevalence= 2.1%; *C. magnus*, prevalence= 1.03%), may be higher than recorded in this study, given that nasal swabbing and culturing has not been standardised in *S. brachyurus*, therefore it is possible that cryptococcal cells may have been missed during the procedure, and positive animals may have been recorded as false negatives. Although *C. neoformans* var. *grubii* may have been recovered as a result of transient contamination, it is unlikely given that known environmental sources were not present at the moment of sampling, and all animals were sampled under the same conditions.

Cryptococcus neoformans (var. *neoformans* and var. *grubii*) has a world-wide distribution and an important reservoir of the organism is in bird excreta, particularly that of pigeons (*Columba livia*) (Casadevall & Perfect 1998 ; Li *et al.* 2012), however, studies suggest that other birds may also be natural reservoirs for *C. neoformans* var. *grubii* (Cafarchia 2012). An example of this, is how seabirds have been proposed as a source of cryptococcal infection in dolphins, with the transmission of the fungi occurring while both hosts fed on schools of fish (Gales, Wallace, & Dickson 1985 ; Migaki, Gunnels, & Casey 1978). From a microbiological perspective, these organisms could grow on excreta of any avian species due to the high concentrations of creatinine that are present (Levitz 1991 ; Quinn *et al.* 2011 ; Sperber 1960). Although var. *grubii* has been recovered from a variety of hosts (see Table 5-1), they are generally not considered a source of it. Additional environmental sources include tree debris of *Eucalyptus camaldulensis* (Nishikawa *et al.* 2003 ; Pfeiffer & Ellis 1993), domestic dust and decaying wood (Nishikawa *et al.* 2003).

According to a study by Saunders and Rebeira (2009), 26 specimens of *Columba livia* (an introduced species to Australia) were recorded in two visits to Rottnest Island between 1980 and 2007. The birds were observed in woodland habitats and the Settlement. Even though recent reports on the presence of *C. livia* on Rottnest Island were not obtained, according to the Rottnest Island Authority (Shane Kearney *pers. comm.* 2015), no actions have been taken to control *C. livia* numbers, and it is believed the species still present on the island. In contrast, on the mainland, *C. livia* has been reported mainly around urban areas, away from the forest ecosystems used by *S. brachyurus* (Atlas of Living Australia 2013 ; Department of Parks and Wildlife (DPaW) 2007). Although further studies are required to conclusively determine that *C. livia* does not shares the same ecosystems as the mainland quokka, previous studies have shown that *C. livia* prefers urban environments and open agricultural areas (Birdlife Australia 2015). The preference of *C. livia* for urban ecosystems may be a factor in the reduced or lack of exposure of the quokka to spores of desiccated cells of *C. neoformans* var. *grubii* and/or var. *neoformans* on the mainland, and may have contributed to the negative results in this study.

By simple observation, quokkas in areas around Thompson's Bay settlement (see Figure 5-4) on Rottnest Island appear to be more likely to get exposed to *C. neoformans* var. *grubii* yeasts or spores by scavenging for food sources in areas contaminated with avian excreta from *C. livia* or other species, as well as debris from potential plant sources. However, this could be a case of risk of exposure, and not a case of complete absence of avian reservoirs on the mainland sharing the same ecological niche with *S. brachyurus*. Risk of exposure of *S. brachyurus* to bird droppings appears to be greater on the settlement areas of Rottnest

Island than on any other location of the natural range of the species, not just on the island but also on the mainland.

Some plants and trees have been reported to be a niche for *C. neoformans* var. *grubii*: *Cassia grandis, Eucalyptus camaldulensis, Ficus microcarpa*, *Myroxylon peruiferum, Senna multijuga*, *Syzygium jambolanum*, and *Theobroma cacao* (Nishikawa *et al.* 2003), as well as *Moquilea tomentosa* (Granados & Castañeda 2005). Of these, *Ficus microcarpa* and *Eucalyptus camaldulensis* are the only ones that have been recorded in WA (Atlas of Living Australia 2013 ; Western Australian Herbarium 1998). *E. camaldulensis* is the only species that has been reported on the mainland with just a few observations within the natural range of *S. brachyurus*, whereas both *F. microcarpa* and *E. camaldulensis*, (considered introduced species), have been described as been concentrated in areas of Rottnest Island that have been urbanised (Rippey, Hislop, & Dodd 2003). Even though sampling of environmental sources was not part of this study, there appears to be a potential association between the isolation of *C. neoformans* var. *grubii* from the nasal lining of *S. brachyurus* present in urbanised areas of Rottnest Island, and the possible higher incidence of *C. livia, F. microcarpa* and *E. camaldulensis* in these same areas of the island.

The negative results for *C. neoformans* (both varieties) from nasal swabs of mainland animals may be explained by a number of factors, including: i) a small sample size (n= 33), ii) the limited number of sites where animals were trapped (n= 3), iii) the limited presence or absence of environmental sources of *C. neoformans* var. *grubii* (*C. livia*, *E. camaldulensis* and *F. microcarpa*) within the mainland range of the species, and iv) host immunity. The first two factors may have also played a role in the negative results for *C. gattii* from mainland samples. However, unlike *C. neoformans* var. *grubii* environmental sources that appear to be absent from the natural range of *S. brachyurus* on the mainland, *Eucalyptus rudis* a known environmental niche for *C. gattii* (Connolly *et al.* 1999 ; Pfeiffer & Ellis 1997), has a widespread presence across the mainland range of the species (Atlas of Living Australia 2013 ; Department of Parks and Wildlife (DPaW) 2007 ; Western Australian Herbarium 1998). *C. gattii* has been previously recovered from the wood, bark, leaves and flowers collected under the canopy of *E. rudis* (Connolly *et al.* 1999). Although further studies are needed to corroborate the relation of *E. rudis* and *C. gattii* within the mainland range of *S. brachyurus,* the absence of *C. gattii* in mainland samples may reflect that: i) animals may not be in direct contact or may have limited exposure to wood, bark, leaves or flowers of *E. rudis*, and ii) that this may have to do with the thick understory and the use of runnel systems that quokkas use to move from one point to another. These two factors may be serving as barriers that decrease animal exposure to the organisms.

Although clinical signs of cryptococcosis may vary depending on the organs affected, some of which are only assessable by diagnostic techniques not easily available in the field (e.g. ultrasound, X-ray imaging or even high definition imaging techniques such as computed axial tomography), animals in this study were considered to be apparently healthy at the time of sampling. There were no typical signs of cryptococcosis by *C. neoformans* (e.g. neurological, granulomas in nasal cavity, peripheral lymphadenopathy, respiratory like sneezing or coughing, Vogelnest & Woods 2010), no association between physical examination findings and the presence of *Cryptococcus* spp., and no significant differences in the HMT and BLC between *Cryptococcus* spp.-positive and *Cryptococcus* spp.-negative animals. This agrees with previous studies in marsupials (Connolly *et al.* 1999 ; Krockenberger *et al.* 2002), domestic animals (Malik *et al.* 1997), pigeons and cormorants (Danesi *et al.* 2014), that have shown that *C. neoformans* var. *grubii* could be carried asymptomatically. However, it is worth noting, that the negative culture results, coupled with the absence of statistical effect of *Cryptococcus* spp. on blood parameters, and the absence of significant associations with physical examination findings, are not sufficient to differentiate between asymptomatic carriers and animals with early stages of cryptococcosis (e.g. early lesions on the mucosa of the cribriform plate). The effect of sex on the BLC of Rottnest Island animals may be attributed to the interplay of multiple cofounding factors such as age, nutrition or genetic differences.

Despite the absence of obvious clinical signs of disease at the time of sampling, and the absence of any significant differences between the HMT, BLC and PBCM of *C. neoformans* var. *grubii* positive and negative animals, we recommend these results be interpreted with caution. Inhalation of aerosolised cryptococcal cells (i.e. yeast cells or basidiospores) desiccated by environmental exposure is believed to be the most likely route of infection (Sykes & Malik 2012). *C. neoformans* var. *grubii* cells in turn, could progress into silent primary foci in the respiratory passages and organs (Merchant & Packer 1967 pp. 549-566), that under the right conditions (e.g. stress-induced immunosuppression) can reactivate and become the source of systemic dissemination, that may involve the central nervous system, bone tissue, skin or other organs (Garcia-Hermoso, Janbon, & Dromer 1999 ; Illnait-Zaragozi *et al.* 2011 ; Krockenberger *et al.* 2002). The presence of *C. neoformans* var. *grubii* in the nasal cavity of quokkas on Rottnest Island, suggests that inhalation of aerosolised cryptococcal cells could follow, or has already occurred. This is relevant for *ex-situ* and *in-situ* management programs of *S. brachyurus*. To the best of our knowledge, captive populations of *S. brachyurus* throughout Australia are populated by specimens that were sourced from Rottnest Island. Consequently, it is reasonable to considered that animals captured in urbanised areas of the island, may have arrived at

their captive collections carrying basidiospores or having developed silent cryptococcal lesions in their lungs, and are at greater risk of developing clinical disease. Even though further studies would be necessary to determine whether *C. neoformans* var. grubii-positive animals acquired the infection prior to their captivity, and whether reactivation of dormant cryptococcal lesions occurs in *S. brachyurus*, it is suggested that in addition to standard cryptococcal tests such as latex cryptococcal agglutination test (LCAT), advanced imaging techniques such as computed axial tomography or magnetic resonance imaging are considered to screen for *pulmonary lesions* consistent with cryptococcal pathology in new arrivals. Alternatively, nasal endoscopy should be considered at minimum, to detect lesions in the cribriform plate consistent with cryptococcal pathology.

Pertaining to the population on Rottnest Island (i.e. *in-situ*), even though *S. brachyurus* and *C. neoformans* var. *grubii* may have co-evolved in the same natural environment (a relation that commonly does not lead to disease), certain conditions such as exposure to large amounts of infective yeast cells, and a compromised immune system (e.g. stress-induced immunosuppression), could alter the balance host-parasite relation, which could then lead to cryptococcal disease. Although there is insufficient evidence (e.g. from histological examination, high definition imaging techniques) to prove that silent pulmonary or upper respiratory cryptococcal foci are present in animals on Rottnest Island, we believe it is possible that such lesions are present in animals that have been in contact with *C. neoformans* var. *grubii*. Consequently, animals with such lesions may be at a greater risk of disease, particularly in the presence of concomitant disease and chronic stress due to increasing environmental pressures such as climate change, habitat loss, and food and water shortage. Even though *C. neoformans* var. *grubii* is not known to be transmitted from animal to animal (Cafarchia 2012 ; Vogelnest & Portas 2010 pp. 133-225), hence disease should be expected only in animals that have been exposed to the environmental source of *C. neoformans* var. grubii. However, a study by (Krockenberger, Canfield, & Malik 2002) suggested that heavily colonised or infected koalas could contaminate previously cultured-negative vegetation. Consequently, moving animals from Rottnest Island to the mainland, may represent a potential biological hazard to mainland individuals.

Cryptococcosis is no longer considered an opportunistic infection in human health, and today, is one of the major diseases of medical importance in both immunocompetent and immunocompromised individuals, particularly children (da Costa *et al.* 2013 ; Del Poeta & Casadevall 2012), with an estimated 650,000 deaths annually (Pappas 2013). Therefore, from a public health point of view, the isolation of *C. neoformans* var. *grubii* from *S.*

brachyurus on Rottnest Island requires further attention. Two main aspects should be considered: i) the possible zoonotic transmission from *S. brachyurus* to humans, and ii) the risk of exposure of people to *C. neoformans* var. *grubii* environmental source (sources). To our knowledge, the quokka has been associated with the spread of agents capable of disease, particularly *Salmonella*. In 1973, a child was diagnosed with salmonellosis due to an infection with *S. enterica* ser. Javiana, that was acquired after the child handled faecal pellets on Rottnest Island (Iveson & Bradshaw 1973). Pertaining *C. neoformans,* to the extent of our knowledge only pet birds have been implicated in cases of zoonotic transmission to humans (Lagrou *et al.* 2005 ; Nosanchuk *et al.* 2000). However, studies in mice (immunosuppressed) inoculated orally with *C. neoformans* (variety not specified), detected intestinal shedding of viable yeast cells for up to 12 months (Green & Bulmer 1979 ; Salkowski *et al.* 1987). Although further studies would be necessary to establish whether faecal shedding of viable *C. neoformans* yeast cells by *S. brachyurus* is possible, ingestion of *C. neoformans* var. *grubii* by animals on Rottnest Island through direct contact with avian excreta or another environmental source (e.g. *F. microcarpa* and *E. camaldulensis*) while scavenging for food, is likely. In the interest of public health and animal management, it is advisable to consider faecal shedding of *C. neoformans* var. *grubii* by *S. brachyurus* on Rottnest Island possible, until demonstrated otherwise.

The presence of an environmental source (sources) on Rottnest Island, supported by the recovery of *C. neoformans* var. *grubii* from the nasal passages of *S. brachyurus*, represents a risk factor for cryptococcal disease in humans. Consequently, the Rottnest Island Authority and institutions responsible for public health are advised to considered exposure to *C. neoformans* var. *grubii* as likely. Further studies would be necessary, to conclusively determine what the environmental source (sources) for *C. neoformans* var. *grubii* on Rottnest Island is, and how humans interact with this source (sources). This would allow to design and carry out strategies aimed to decrease the overall risk of cryptococcal disease in humans.

The outbreak and emergence of *C. gattii* in Vancouver Island (Canada) that was detected in 2002 (Hoang *et al.* 2004), is an example of pathogen adaptability to new environments and the importance of disease surveillance. *C. gattii* was known to be associated with tropical and semi-tropical environments, however, the organism thrived in the temperate climate of VI (Kidd *et al.* 2004). Although cases of cryptococcosis had been happening since 1997, cases were considered to be isolated, and no epidemiological studies were performed until disease incidence had reached levels higher than that recorded in Australia, were *C. gattii* is endemic (Kidd *et al.* 2004). In these previous studies, attention

to other potentially susceptible hosts (e.g. wildlife) was not given. It was Duncan et al. (2006) who isolated *C. gattii* from the nasal passages of eastern grey squirrels (*Sciurus carolinensis*) in Vancouver Island. Whether *C. neoformans* var. *grubii* was introduced or is endemic to Rottnest Island, is difficult to determine as the agent has not been surveyed in the past. However, testing potentially susceptible hosts other than humans (e.g. *S. brachyurus*), that may be constantly exposed to the environmental source (sources) of *C. neoformans* var. *grubii*, appears as a possible environmental indicator of human risk. The recovery of live *C. neoformans* var. *grubii* from the nasal cavity of *S. brachyurus* on Rottnest Island, emphasises the importance and need of surveillance studies of wildlife health, highlights the potential role of *S. brachyurus* as a sentinel species, and is a voice of alert to public health agencies. The impact of *C. neoformans* var. *grubii* on the quokka and other wildlife species on Rottnest Island remains largely unknown and warrants further investigation.

Cryptococcus magnus, *Rhodosporidium kratochvilovae*, *Rhodotorula mucilaginosa*, *Rhodotorula glutinis* and *Aureobasidium pullulans* are considered to be ubiquitous with no specific ecological niches or natural hosts. Although they have been previously associated with diseases in different animal species (Beemer, Schneerson-Porat, & Kuttin 1970 ; Kadota *et al.* 1995 ; Monga & Garg 1980 ; Poth *et al.* 2010 ; Wirth & Goldani 2012b) and in humans (Clark *et al.* 1995 ; de Oliveira *et al.* 2013 ; Huttova *et al.* 1998 ; Wirth & Goldani 2012a), they are not typically considered to be highly pathogenic. In humans, disease conditions are generally associated with environments in which patients are subject to invasive procedures (e.g. central catheterisation and surgery) that serve as an entry point for these ubiquitous yeast and as with cryptococcal infection, in immunosuppressed patients. In animals, it appears that the nosocomial source of infection has not been reported for any of these yeast as yet, and even though these organisms have been recovered from animals with a variety of conditions, the prevalence and incidence of these seem to be very low. For instance, there is only one case of cryptococcosis in cats due to *C. magnus* in the literature (Poth *et al.* 2010). Considering the low incidence and prevalence of these yeast, as well as the mild presentation of the multiple clinical conditions (e.g. dermatitis, epididymitis) reported in the literature, it appears reasonable to consider these organisms as of little concern in the context of *S. brachyurus* conservation. However, it is important to keep present that under immunosuppressive states, animal models of disease have shown that these yeast, that are not typically pathogenic, have the ability to cause multisystemic disease (Wirth & Goldani 2012b).

6. A novel gammaherpesvirus in free-ranging quokkas (*Setonix brachyurus*) on Rottnest Island and mainland Western Australia

STATEMENT OF AUTHOR CONTRIBUTION

P. Martinez-Perez: designed the general scope and structure of the chapter, initiated the research, carried out all fieldwork procedures, carried out all laboratory procedures for the molecular detection and characterisation of MaHV-6, performed blood smear assessment, entered and analysed the data, and wrote the chapter.

P.A. Fleming: advised and assisted with statistical analyses, and provided editorial comments to versions of the chapter from draft to final version.

T.H. Hyndman: provided training and advised in PCR methods and bioinformatics, and provided editorial comments to versions of the chapter from draft to final version.

C.R. Wilks: coordinated serology testing, and provided editorial comments to versions of the chapter from draft to final version.

6.1 Abstract

In their natural hosts, herpesviruses may cause disease that is rarely fatal, but can pose a significant threat to other closely related host species. To date, five herpesviruses have been reported in macropods. In this study, we screened 142 free-ranging quokkas (*Setonix brachyurus*) from Rottnest Island and three sub-populations on the mainland (mainland) for herpesvirus infection by a pan-herpesviral nested polymerase chain reaction (nPCR) or by serological testing for neutralising antibodies to MaHV-1 and MaHV-2. We compared these data with haematology (HMT), blood chemistry (BLC), peripheral blood cell morphologies (PBCM), clinical status, and other concomitant infections. Neutralising antibodies to MaHV-1 or MaHV-2 were detected in one free-ranging individual from the mainland of Western Australia (WA) (prevalence= 0.7%, 95% CI 0.1-3.2). DNA of a novel gammaherpesvirus (designated MaHV-6) was detected by nPCR in the blood of 13 of 121 (prevalence=10.7%, CI 6.2-17.2) apparently healthy animals (combined sample Rottnest Island and mainland). This novel macropodid herpesvirus is related to, but phylogenetically distinct from previously reported gammaherpesviruses in macropods. There was no association between MaHV-6 infection and changes in HMT, BLC or PBCM, and there was no association between the presence of MaHV-6 infection and

physical examination findings for these animals. There was significantly (p= 0.015) greater prevalence of MaHV-6 on the mainland (25%, n= 28, CI 12.7-43.4) compared to Rottnest Island (6.45%, n= 93, CI 2.7-12.8). There was a significant association between the presence of *Theileria* sp. in peripheral blood (p=0.001) and the presence of MaHV-6, with MaHV-6 positive animals being 11 times more likely to be infected with *Theileria* sp. (OR= 11.0, CI 2.31-52.3). This may suggest that quokkas may be more susceptible to infection with *Theileria* sp., if infected with MaHV-6.

6.2 Introduction

Members of the family *Herpesviridae* are enveloped, and have double-stranded deoxyribonucleic acid (DNA) genomes inside an icosadeltahedral capsid, with virions reaching almost 300 nm in diameter (Widén *et al.* 2012). The family is subdivided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae*, and *Gammaherpesvirinae* (Davison *et al.* 2009). Herpesviruses are of great significance in veterinary medicine; they are responsible for at least one major disease in each species of most domestic animals (MacLachlan & Dubovi 2011). This significance also extends to captive and free-ranging wildlife (King 2001), where herpesviruses have been responsible for mortality in captive collections and mass mortalities in wild fish (Garver *et al.* 2010 ; Jones *et al.* 1997).

In the natural host, herpesviruses may cause disease that is rarely fatal, and following recovery, animals usually establish lifelong infection characterised primarily by periods of latency in various tissues with intermittent recrudescence of infectious virus (Roizman & Pellet 2001). Latency is often in nervous tissue, however, gammaherpesviruses in particular are lymphotropic in nature, establishing latency primarily in either B- or T-lymphocytes (Markey *et al.* 2013 pp. 559-573). Periodic recrudescence with virus shedding is sometimes accompanied by clinical signs of disease. By contrast, herpesviruses that are transmitted from their natural host to another host species (frequently a related species) often cause fatal disease (Roizman & Pellet 2001). A classic example of this is wildebeest-associated malignant catarrhal fever, a disease caused by a gammaherpesvirus (*Alcelaphine herpesvirus* 1). This virus is carried by wildebeest (*Chonnochaetes taurinus* and *C. gnu*) asymptomatically, but reliably causes fatal generalised disease in susceptible species such as cattle, bison and deer (Markey *et al.* 2013). A similar example is B-virus (*Cercopithecine herpesvirus* 1) of macaques (*Macaca* spp.) that usually results in fatal disease if transmitted to humans (Huff & Barry 2003). It is believed that every vertebrate species is host for at least one species of herpesvirus (MacLachlan & Dubovi 2011 ; Portas *et al.* 2014). New members of each *Herpesviridae*

subfamily have been recently detected in a broad range of wildlife species, including vespertilionid bats (*Miniopterus fuliginosus*) (Watanabe *et al.* 2010), common loons (*Gavia immer*) (Quesada *et al.* 2011), fishers (*Martes pennanti*) (Gagnon *et al.* 2011), northern sea otters (*Enhydra lutris kenyoni*) (Tseng *et al.* 2012), bobcats (*Lynx rufus*) and pumas (*Puma concolor*) (Troyer *et al.* 2014), and belugas (*Delphinapterus leucas*) (Bellehumeur *et al.* 2015), among others. Furthermore, studies have also shown that several species of herpesviruses could be present concomitantly in the same animal, for instance: elephant endotheliotropic herpesvirus 1 and 5 in free-ranging Asian elephants (*Elephas maximus*) (Stanton *et al.* 2014) and phascolarctid herpesvirus 1 (PhaHV-1) and 2 (PhaHV-2) in free-ranging koalas (*Phascolarctos cinereus*) from eastern Australia (Vaz *et al.* 2012). However, of these recent discoveries, gammaherpesviruses appear to be the most frequent.

Infections with herpesviruses have been reported in members of the family Macropodidae (kangaroos and wallabies). Infection with *Macropodid herpesvirus 1* (MaHV-1) an alphaherpesvirus was detected in captive parma wallabies (*Macropus parma*) (Acland 1981 ; Finnie, Littlejohns, & Acland 1976) and was associated with respiratory signs, conjunctivitis, incoordination, pyrexia and ulcers in the cloacal and pericloacal regions (Acland 1981 ; Finnie, Littlejohns, & Acland 1976). Infection with *Macropodid herpesvirus 2* (MaHV-2; also an alphaherpesvirus) was detected in captive Dorcopsis wallabies (*Dorcopsis muelleri luctuosa*) and in a male quokka (*Setonix brachyurus*) in a mixed-species enclosure at Melbourne Zoo (Callinan & Kefford 1981 ; Wilks, Kefford, & Callinan 1981). The isolates were initially identified as Dorcopsis wallaby herpesvirus and Quokka herpesvirus respectively; however, restriction endonucleases studies subsequently unified them into MaHV-2 (Johnson & Whalley 1987 ; Johnson *et al.* 1985). Although infection in both species was somewhat similar, oral ulcers and persistent bilateral purulent conjunctival exudate were the main clinical signs in the infected male quokka that was found dead 24 h after oral and cloacal ulcers were observed (Callinan & Kefford 1981 ; Wilks, Kefford, & Callinan 1981).

Despite the presence of several other species at the time, fatal disease was only seen in *S. brachyurus* and *D. m. luctuosa*. Consequently, it was suggested that the outbreak at Melbourne Zoo may have been a case of MaHV-2 transmitted from another macropod species, possibly the natural host (Wilks, Kefford, & Callinan 1981) to the two species of macropodids with which it would not normally have contact in the wild. A serological survey was also performed at the time, with sera from nine macropod species held in captivity (except *S. brachyurus*). Neutralising antibodies to MaHV-2 were present in 44.7% of the animals tested (n= 47), similar to previously reported prevalences by

Webber and Whalley (1978) for MaHV-1 in free-ranging and captive marsupials (mostly macropods). More recently, prevalence of neutralising antibodies to MaHV-1 and MaHV-2 in captive and free-ranging animals from eastern Australia, was found to range from 0% (n= 8) in koalas (*Phascolarctos cinereus*), 66.7% (n= 15) in common wombats (*Vombatus ursinus*), to 92% (n= 25) in eastern grey kangaroos (*Macropus giganteus*) (Stalder 2013). Although antibodies to these two alphaherpesviruses appear to be quite prevalent in the Australian macropod populations, it is not known if cases of disease occur in wild populations and if so, what their clinical significances and *natural* hosts are. This suggests, as some authors have proposed, that these viruses have coevolved with marsupial species and are endemic to free-ranging populations in Australia (Webber & Whalley 1978 ; Wilks, Kefford, & Callinan 1981).

Macropodid herpesvirus 3 (MaHV-3; a gammaherpesvirus) was first detected in a captive collection of eastern grey kangaroos (*Macropus giganteus*) in the USA, and similar to MaHV-1 and MaHV-2, detection of the virus occurred in association with disease (although of less severity) and death of some animals. MaHV-3 was then detected by nPCR in a sick free-ranging eastern grey kangaroo (*Macropus giganteus*) in Victoria, Australia (Wilcox *et al.* 2011). The animal survived after treatment, however, although the presence of MaHV-3 was temporally associated with disease (nasal and bilateral serous ocular discharges, pyrexia and respiratory disease), and that other animals in the mob where this animal was part of showed similar clinical signs as well as dead animals, the association could not be shown to be causal (Wilcox *et al.* 2011).

In 2013, a third alphaherpesvirus was detected in a wild eastern grey kangaroo (*M. giganteus*) with clinical signs compatible with respiratory and neurologic disease (Vaz *et al.* 2013). Although closely related to MaHV-1 (94.6% similarity according to glycoprotein B and glycoprotein G amino acid sequence identities) and MaHV-2 (82.7%), genome restriction endonuclease cleavage analysis and cell culture characteristics identified this virus as sufficiently distinct to warrant its classification as Macropodid herpesvirus 4 (MaHV-4) (Vaz *et al.* 2013). A subsequent study on the prevalence of herpesviruses in captive and free-ranging Australian marsupials has detected another novel gammaherpesvirus in free-ranging and apparently healthy swamp wallabies (*Wallabia bicolor*) (prevalence= 26.7%, n= 15) (Stalder *et al.* 2015) which has been named Macropodid herpesvirus 5 (MaHV-5), on the basis of genome sequencing. Infections with herpesviruses that have not been characterised by molecular methods, have also been reported in the red kangaroo (*M. rufus*) (Britt Jr, Frost, & Cockrill 1994) and brush-tailed rock wallabies (*Petrogale penicillata*) (Canfield & Hartley 1992) among others.

In this study, we screened 142 free-ranging *S. brachyurus* from Rottnest Island and the mainland of WA for neutralising antibodies to MaHV-1 or MaHV-2. We also carried out nPCR amplification for herpesviruses and report the detection of a novel gammaherpesvirus in 13 apparently healthy animals.

6.3 Materials and Methods

Between September 2010 and December 2011, 153 quokkas were captured on Rottnest Island and three sub-populations across southwest WA. For trapping sites descriptions and locations, and general procedures, including biological sample collection, anaesthesia and physical examinations, refer to Chapter 3, section 3.3. All data were recorded for each animal and entered into an electronic database (FileMaker© v12, 2013). Blood samples (EDTA and lithium heparin) were collected from the lateral tail vein and stored at 4 ˚C for later processing.

Of the 153 animals trapped, 142 were tested for neutralising antibodies to MaHV-1 and MaHV-2 by the School of Veterinary and Agricultural Sciences at The University of Melbourne. Positive serological results prompted a molecular approach, and 121(n= 93 from Rottnest Island and n= 28 from the mainland) of the 142, were then tested for herpesviruses using DNA from whole blood, at Murdoch University. Virus neutralisation testing methods were followed (as per Vaz *et al.* 2013).

Complete blood counts and BLC panels were performed at the Clinical Pathology service of the Murdoch University Veterinary Hospital. The following HMT parameters were recorded: white blood cell counts (corrected to exclude nucleated red blood cells) (WBC), red blood cell concentration (RBC), haemoglobin concentration (HGB), packed cell volume (PCV), the absolute concentrations for neutrophils (NEUT), eosinophils (EOS), basophils (BASO), lymphocytes (LYMPH) and monocytes (MONO). The following BLC analytes were tested: alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), total protein (PROT), albumin (ALB), calcium (CA), phosphorus (P), cholesterol (CHOL), total bilirubin (BILT), glucose (GLUC), creatinine (CREAT), urea (UREA), and vitamin E (Vit E). Blood smears were assessed manually (see Chapter 3, section 3.4.2) to determine the differential leukocyte count (200 leukocytes), polychromatophilic erythrocyte count (1,000 erythrocytes), and PBCM (e.g. reactive lymphocytes, atypical neutrophils, keratocytes, schistocytes). Microfilariae were recorded from blood smears, while *Theileria* sp. and *Babesia* sp. were screened for nPCR (see Chapter 7, section 7.3.1). Nematode eggs and *Eimeria* sp. oocysts were screened by

light microscopy (see Chapter 3, section 3.4.1). *Cryptococcus* spp. were screened by culture methods and isolates characterised by PCR (see Chapter 5, section 5.3.1), while *Salmonellae* spp. were screened on stool samples by culture methods and identified by serotyping (see Chapter 4, section 4.2.1).

6.3.1 *DNA extraction, amplification and sequencing*

DNA was extracted from blood samples using either the QIAamp® DNA Mini Kit (QIAGEN®, Hilden, Germany) or the ISOLATE II Blood DNA Kit (Bioline Reagents Ltd., London, United Kingdom) (see Appendix 10.3.1 for detailed description of the DNA extraction methods). All DNA products were then tested by nPCR for the presence of herpesviruses (as per VanDevanter *et al.* 1996) (see Appendix 10.3.2 for detailed description of the nested PCR methods), targeting the DNA-dependant polymerase (DPOL) gene, a highly conserved region of the viral genome and is one of the more commonly sequenced herpesvirus genes; these features make the DPOL gene well suited to genetic comparisons of herpesvirus species (Pellet & Roizman 2007). Reactions were then visualised in agarose gel. *Equine herpesvirus* 1 (EHV-1) was used a positive control. Bands of the expected size were cut out using sterile scalpel blades and DNA was purified from agarose using the filter tip method (see Appendix 10.3.2). Purified nPCR products were then sequenced (see Appendix 10.3.2.1 for details) using the forward and reverse primers on an ABI 3790 96 capillary automatic sequencer (Applied Biosystems, Scoresby, Australia). Primer sequences were removed and the remaining sequence information was analysed using 4Peaks version 1.7.2 (A. Griekspoor and Tom Groothuis, at http://nucleobytes.com/index.php).

6.3.2 *Phylogenetic analysis*

Nucleotide sequences were translated, and open reading frames were found using ExPASy Bioinformatics Resource Portal (http://web.expasy.org/translate/). The predicted amino acid sequence was compared with sequences in GenBank (http://www.ncbi.nlm.nih.gov/) using BLASTP v. 2.2.29+. Predicted amino acid sequences were aligned with homologous sequences of herpesviral DNA-dependant-DNA polymerase obtained from 33 different host species, representing the three *Herpesviridae* subfamilies (*Alpha-*, *Beta-*, and *Gammaherpesvirinae*) . Sequences were aligned using MUSCLE (Edgar 2004). Phylogenetic reconstruction of the aligned sequences was performed using MEGA5 v. 5.2.2 (Tamura *et al.* 2011). To identify the best amino acid substitution model, initial trees for the heuristic search were obtained by applying the Neighbour-Joining method to a matrix

of pairwise distances estimated using a Jones-Taylor-Thorton model. The evolutionary history was inferred using a maximum likelihood method, running a Le-Gascuel amino acid substitution model (Le & Gascuel 2008). A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories; +G, parameter= 1.5314). The rate variation model allowed for some sites to be evolutionarily invariable (+1/; 19.4% sites). Lastly, an unrooted maximum likelihood tree was generated. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. The strength of each tree branch was assessed using a bootstrapping analysis with 200 replications (Efron, Halloran, & Holmes 1996).

6.3.3 *Statistical analyses*

Haematology, BLC, and PBCM datasets for animals that tested positive or negative by nPCR to MaHV-6, were explored visually with non-metric Multidimensional Scaling (nMDS) using a Bray-Curtis similarity measure (Bray & Curtis 1957) in PAST v. 3.02 (Hammer, Harper, & Ryan 2001). Analyses were performed on the three datasets separately due to differences in sample sizes between them (some individuals were tested for either HMT or BLC, which would mean their exclusion from analyses of the entire dataset due to missing data). This analysis was carried out for Rottnest Island and mainland populations combined including MaHV-6 PCR-positive or MaHV-6 PCR-negative, *site* (Rottnest Island or mainland), and *sex* as independent factors. Due to insufficient observations as well as limitations of the multivariate analysis test used, seasonal differences were explored using χ^2 for Trend.

Haematology and BLC dependant variables were range-standardised to a scale between 0 and 1, while PBCM variables had a binary format. For each nMDS plot, two-or-three-dimensional analyses were selected according to the model that had the lowest stress statistic to determine adequacy of the fit. To determine the similarity or dissimilarity of HMT, BLC and PBCM datasets between the groups of interest (MaHV-6 PCR-positive and MaHV-6 PCR negative), a pairwise similarity percentage (SIMPER, PAST v. 3.02) analysis (Clarke 1993) using the Bray-Curtis similarity measure (Bray & Curtis 1957) was carried out. SIMPER results (i.e. percent of contribution of each variable to the similarity or dissimilarity) are accompanied by the arithmetic mean ($\bar{x}$) and standard deviation (SD) for each HMT and BLC variable, while odds ratio (OR) and 95% confidence intervals (CI) were calculated (Woolf 1955) for each PBCM observed in blood smears. To test for statistical significance of these relationships, a two-way analysis of similarity (ANOSIM) (Clarke

1993) with 9,999 permutations for *R* statistics was run (PAST v. 3.02). The *R* test statistic used by ANOSIM ranges from -1 to 1, with $R < 0$ indicating that the dissimilarity of the measured variables (HMT, BLC and PBCM) within groups is greater than between groups, and $R > 0$ indicates greater dissimilarity between groups than within groups.

Chi-square with Yate's correction was used to explored differences in MaHV-6 prevalence by *sex*, *season* and *site* (Rottnest Island or the mainland), as well as possible associations between physical examination findings and concomitant infections (i.e. *Babesia* sp., *Eimeria* sp., *Salmonella* spp., *Theileria* sp., *Cryptococcus* spp., microfilariae, trypanosomes and nematode eggs) with the presence of MaHV-6. OR and 95% CI for these analyses were calculated using the Woolf's method (1955). For all OR and 95% CI calculations, 0.5 were added to each observed value when a null value was present in a contingency table (Altman 1999). All other 95% CI for estimates of proportions (i.e. prevalence), were calculated using the Wilson model for $n \leq 40$, and the Jeffreys model for $n \geq 40$ (Brown, Cai, & DasGupta 2001). Sex differences at individual locations on Rottnest Island were explored with Fisher's exact test. Significance was set at $p < 0.05$ for all statistical analyses.

6.4 Results

6.4.1 *Serology*

Evidence of antibodies against MaHV-1 (titre[12] 128) and MaHV-2 (titre 512) was observed in 1 of the 142 animals tested (prevalence= 0.7%, 95% CI 0.01-0.03). This animal was a male quokka trapped on mainland WA (Armadale). Some neutralisation of the cytopathic effect of MaHV-1 was observed in serum of six animals, while some neutralisation of MaHV-2 cytopathic effect was observed in serum of two more animals. These results, however, were not considered conclusive.

6.4.2 *Phylogenetic analysis*

Herpesviral DNA was detected by nPCR in peripheral blood of 13 of 121 (prevalence= 10.7%, 95% CI 0.06-0.17) quokkas. All shared an identical sequence of 55 predicted amino acids. When compared to homologous sequences on GenBank (http://www.ncbi.nlm.nih.gov/), of the first 100 matches, 94 were from viruses in the *Gammaherpesvirinae* subfamily. Of these 94 sequences, the highest score was for Macropodid herpesvirus 5 [MaHV-5 (Stalder *et al.* 2015), E-value= 3e-30, 76% amino acid

[12] Reciprocal of highest dilution of antiserum neutralising 50% of 100 tissue culture infectious dose of virus calculated by the method of Kärber (1931)

identity, (42/55)] and [MaHV-3, GenBank accession no. ABO61861.1, E-value= 2e-29, 73% amino acid identity, (40/55)]. This herpesvirus is herein referred to as MaHV-6 and alignment of all homologous sequences with MUSCLE alignment is shown in Figure 6-1. A maximum likelihood tree using this alignment is shown in Figure 6-2. The obtained predicted amino acid sequences were submitted to the DNA Data Bank of Japan under the accession numbers LC137002-14.

Figure 6-1 MUSCLE alignment of homologous predicted partial herpesviral DNA-dependent DNA polymerase amino acid sequences. Sequence for Macropodid herpesvirus 6 (MaHV6, novel sequence is in bold). Greyed out boxes indicate amino acid substitutions when compared to MaHV5 and MaHV3. *Herpesviridae* subfamilies are indicated to the right of the alignment: *Gammaherpesvirinae* (red vertical line), *Betaherpesvirinae* (blue vertical line) *and Alphaherpesvirinae* (purple vertical line). Accession numbers for sequences obtained from GenBank are: Alcelaphine HV-1 (ALHV1, AAC58060.1), Ateline HV-2 (AtHV2, AAC55644.1), *Babyrousa babyrussa* rhadinovirus 1 (BbabRhV-1, AAO46907.2), Bovine HV-4 (BoHV4, AAK07928.1), Bat gammaHV (BatGHV1, ACY82599.1), Elephantid HV-5 (ElHV5, ABK41480.2), Equid HV-2 (EHV2, AAC55648.1), *Hylobates leucogenys* rhadinovirus 2 (HleuRhV2, AAS17748.1), Macropodid HV-3 (MaHV3, ABO61861.1), Mustelid HV-1 (MusHV1, AAM62282.1), Procavid HV-1 (PrHV1, ABK41481.1), *Rattus tiomanicus* rhadinovirus 1 (RtioRHV1, ABN49963.1), Callitrichine HV-1 (CallitrichineHV1, AAC55645.1), Tapir HV (AAD30142.3), Trichechid HV-1 (TrHV1, ABB54686.2), Blainville's beaked whale gammaherpesvirus (ZiphiidHV1, AAV68930.1), Macropodid HV-5 (MaHV5), Peramelid HV-1 (PeHV1), Aotine HV-1 (AoHV1, AAC55643.1), Cercopithecine HV-5 (CeHV5, AAC55647.1), Human HV-6 (HHV6, NP_042931.1), Murid HV-2 (MuHV2, AAW57296.1), Porcine cytomegalovirus (PCMV, AAF80111.1), Canine HV (CaHV, AAC55646), Chelonid HV-5 (ChHV5, AAL26782.1), Columbid HV-1 (CoHV1, ABP93391.1), FelineHV-1 (FeHV1, AAC55649.1), Gallid HV-2 (GaHV2, AAC55651.1), Macropodid HV-1 (MaHV1), Macropodid HV-2 (MaHV2), Saimiriine HV-1 (SaHV1, AAC55657.1), Suid HV-1 (SuHV1, DAA02153.1), and Vombatid HV-3 (VoHV3). Sequences for MaHV1 and MaHV2 were obtained from Wilcox et al. (2011). Sequences for MaHV5, PeHV1, and VoHV3 were obtained from Stalder et al (2015).

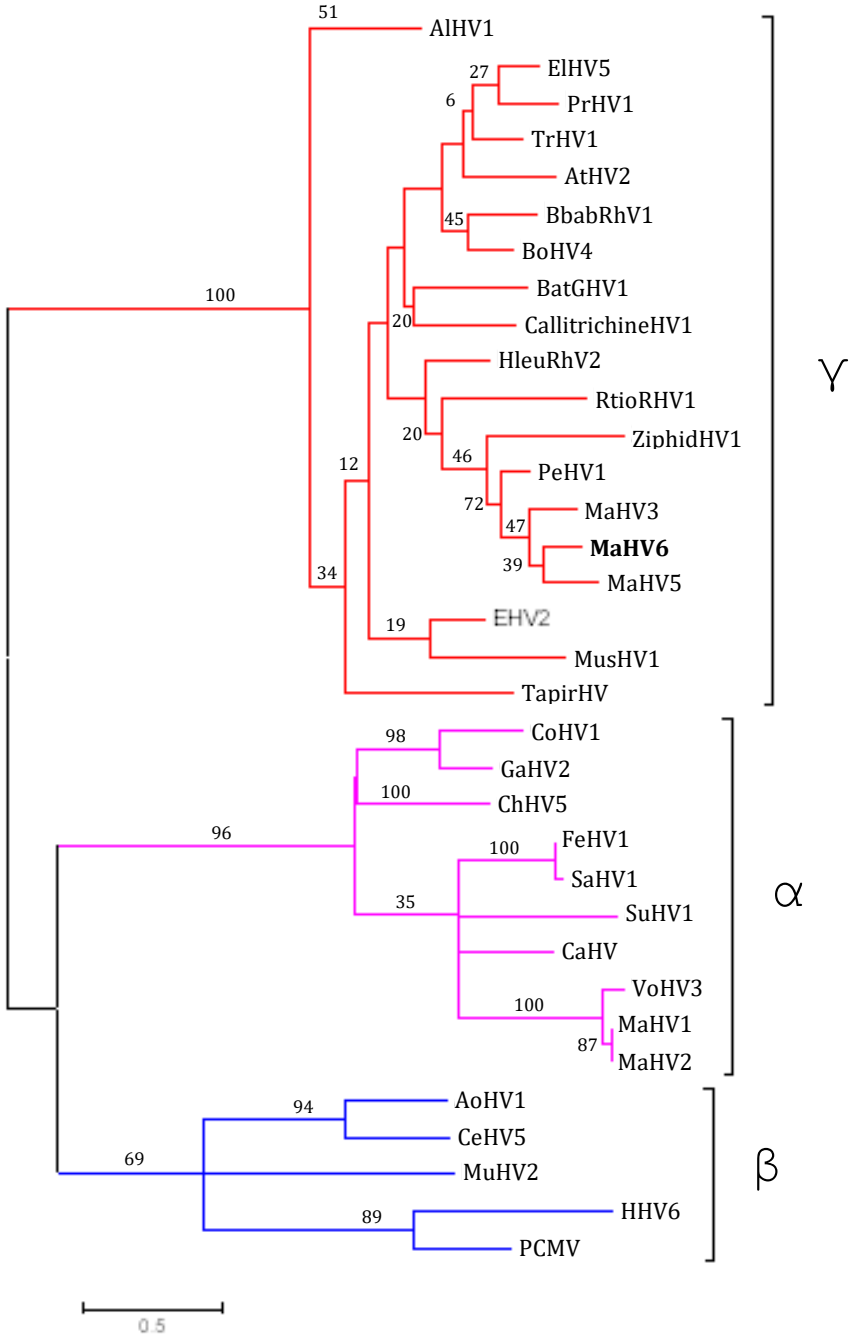


Figure 6-2 Molecular phylogenetic analysis of MaHV-6. The tree (drawn to scale) with the highest log likelihood (-1762.8988) is shown. The percentage of trees in which the associated taxa clustered together is shown above the branches, with branch lengths measured in the number of substitutions per site. The analysis involved 34 amino acid sequences. There were a total of 44 positions in the final dataset. *Herpesviridae* subfamilies are indicated to the right of the alignment: *Gammaherpesvirinae* (γ), *Betaherpesvirinae* (β), and *Alphaherpesvirinae* (α).

6.4.3 *Prevalence and distribution of MaHV-6 on Rottnest Island and the mainland*

Thirteen of 121 *S. brachyurus* from Rottnest Island and mainland sites tested were positive for the presence of MaHV-6 DNA (prevalence= 10.7%, CI 6.2-17.2) (Figure 6-3). There was

a significant difference (χ^2_1= 5.91, p= 0.015) in the prevalence of MaHV-6 in quokkas on Rottnest Island (6/93, 6.45%, CI 2.7-12.8) and that on the mainland (7/28, 25%; CI 12.7-43.4), with mainland animals being 4.83 times more likely to be infected than Rottnest Island animals (95% CI 1.47-15.9). MaHV-6 PCR-positive animals were found at all the three mainland sites, and at half of the Rottnest Island sites (Figure 6-3). There were no seasonal differences in the prevalence of MaHV-6 (χ^2_1= 0.01, p= 0.945) (Figure 6-4).

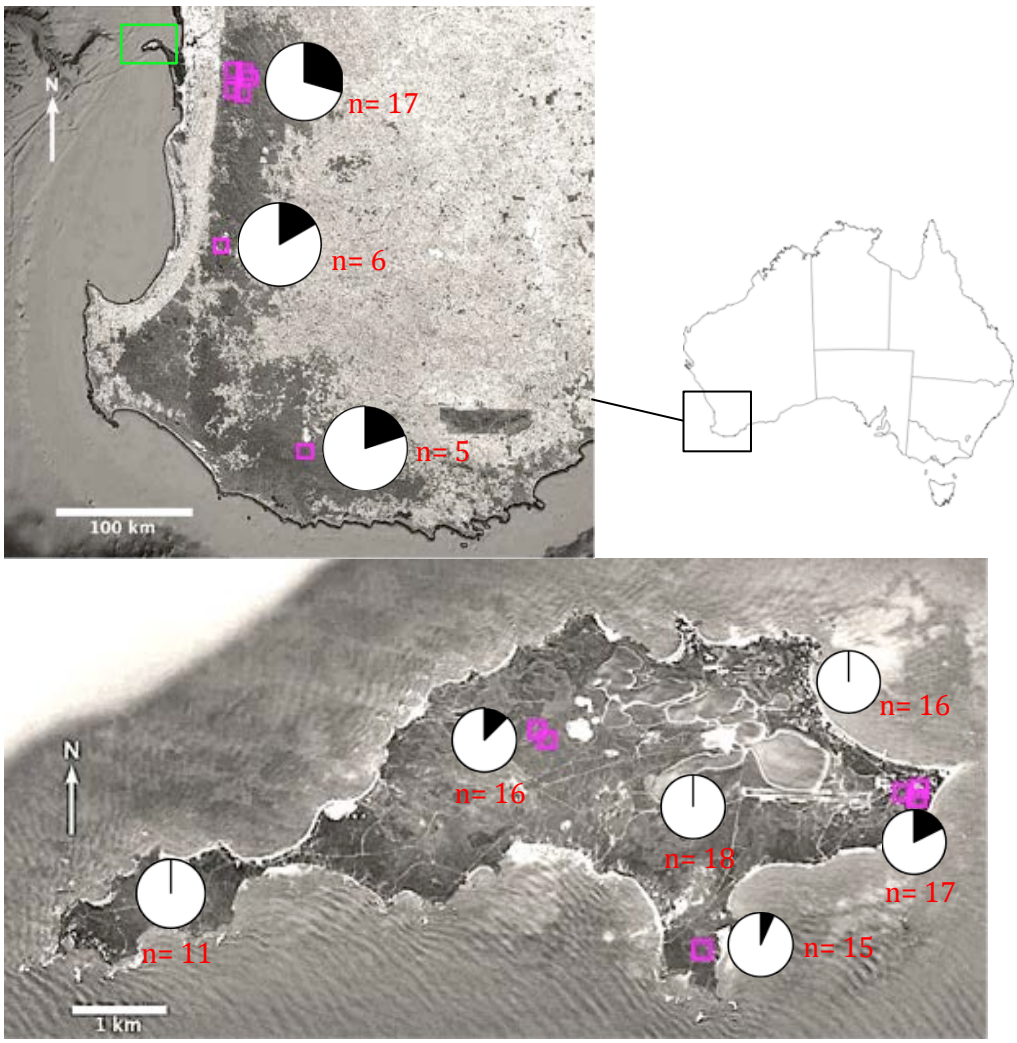


Figure 6-3 Geographic distribution and prevalence (pie charts) of MaHV-6 PCR-positive quokkas from 3 trapping locations on mainland Western Australia (top) and six study sites on Rottnest Island (bottom). Green box indicates the position of Rottnest Island in relation to Western Australia, and purple empty boxes indicate positive animals. Map data ©2016 Google Landsat, Data SIO, NOOA, U.S. Navy, NGA, GEBCO.

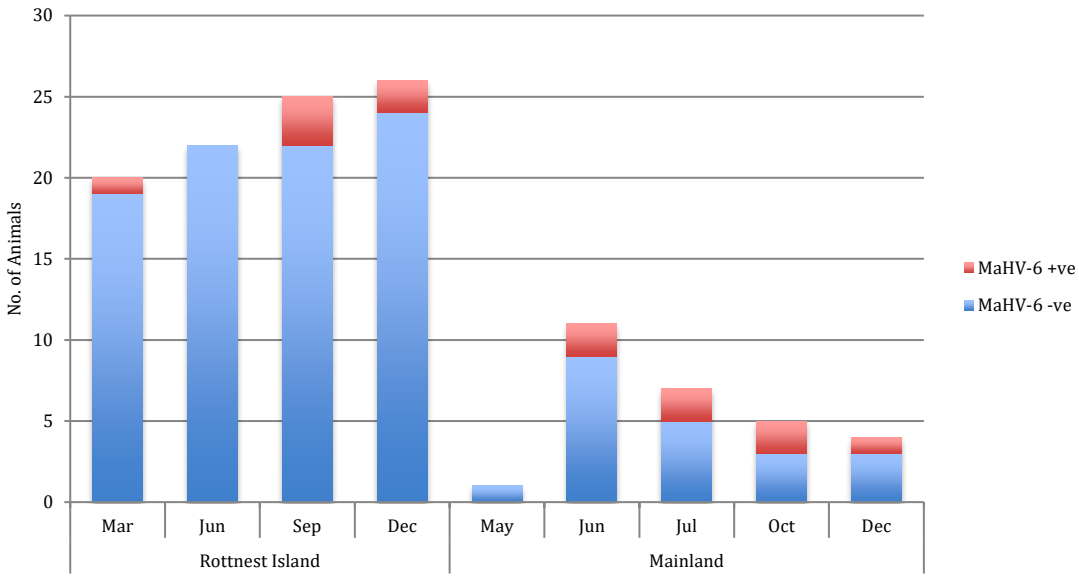


Figure 6-4 Prevalence of MaHV-6 in *S. brachyurus* from Rottnest Island and mainland Western Australia by month of sample.

There was no significant (χ^2_1= 2.36, p= 0.124) difference in the prevalence of MaHV-6 by sex [males 10/74 (13.5%, CI 7.2-0.22.6), females 3/47 (6.38%, CI 1.8-0.16.1)]. Similarly, there was no significant difference in the prevalence of MaHV-6 infection by sex at individual trapping locations on Rottnest Island and on the mainland, where there were sufficient numbers of animals to test this (Table 6-1).

Table 6-1 Comparison between sex and the presence of MaHV-6 DNA in *S. brachyurus* trapped at individual locations on Rottnest Island and the mainland of Western Australia.

Trapping sites	+ve ♂/n (prevalence)	+ve ♀/n (prevalence)	χ^2
Rottnest Island			
Barker Swamp	1/7 (14.3%)	1/9 (11.1%)	$\chi^2_{1,\ n=\ 16}$ = 0.33, p=0.567
Kingston	2/10 (20%)	1/7 (14.3%)	$\chi^2_{1,\ n=\ 17}$ = 0.12, p=0.732
Parker Point	0/8	1/7 (14.3%)	$\chi^2_{1,\ n=\ 15}$ = 0.01, p=0.945
Serpentine	0/10	0/8	-
Settlement	0/9	0/7	-
West End	0/6	0/5	-
Mainland			
Jarrahdale	5/17 (29.4%)	no females sampled	
Collie	1/3 (33.3%)	0/3	$\chi^2_{1,\ n=\ 6}$ = 0.00, p=0.999
Walpole	1/4 (25%)	0/1	$\chi^2_{1,\ n=\ 5}$ = 0.70, p=0.402

6.4.4 *Correlates of MaHV-6 with HMT, BLC, and PBCM variables for Rottnest Island and mainland S. brachyurus*

Subtle differences in the HMT, BLC and PBCM datasets between MaHV-6 positive and negative animals were observed (SIMPER; Table 6-2). Differences were evident in their white blood cell profile with positive animals having a higher count of NEUT, LYMPH, WBC, and greater serum concentrations of CK, AST and ALP, than animals that were negative to MaHV-6.

Table 6-2 Results of SIMPER analyses indicating the contribution of specific variables to the observed differences in HMT (a), BLC (b), and PBCM (c) profiles between animals that were MaHV-6 PCR positive and MaHV-6 PCR-negative (Rottnest Island and mainland combined sample). HMT= haematology, BLC= blood chemistry, PBCM= peripheral blood cell morphology.

		Taxon	Ct %	MaHV-6 +ve		MaHV-6 -ve	
				$\bar{x}$	SD	$\bar{x}$	SD
a.	HMT (26.12) † n +ve= 11 n -ve = 96	Neutrophils (x10.e9/L)	13.3	3.09	1.70	2.28	1.10
		Lymphocytes (x10.e9/L)	12.5	2.04	1.12	1.99	1.12
		Monocytes (x10.e9/L)	11.8	0.06	0.05	0.08	0.07
		RBC (x10.e12/L)	11.4	7.13	1.44	6.11	1.03
		WBC (x10.e9/L)	11.0	5.65	1.46	4.70	1.53
		Basophils (x10.e9/L)	10.4	0.01	0.01	0.02	0.03
		PCV (%)	10.1	39.6	4.86	35	5.15
		HGB (g/L)	9.9	129	22.4	112	18
		Eosinophils (x10.e9/L)	9.6	0.45	0.60	0.32	0.32
b.	BLC (22.39) † n +ve= 11 n -ve= 101	CK (U/L)	10.2	6,636	8,758	2,030	3,547
		AST (U/L)	9.72	300	522	70.1	67
		Vit. E (mg/L)	8.41	7.20	2.33	7.01	2.49
		BILT (µmol/L)	7.98	3.63	1.65	4.48	1.80
		ALT (U/L)	7.91	34	255	260	113
		PROT (g/L)	7.81	65	4.48	61.2	4.9
		ALP (U/L)	7.71	9,242	15,048	7,476	9,224
		PHOS (mmol/L)	7.14	1.47	0.69	1.31	0.56
		UREA (mmol/L)	6.63	9.28	4.01	7.39	2.98
		CALC (mmol/L)	6.60	2.37	0.24	2.25	0.23
		CHOL (mmol/L)	6.26	2.72	00.48	2.84	0.58
		GLUC (mmol/L)	5.74	4.29	1.41	4.39	2.3
		ALB (g/L)	4.66	37.3	1.55	36.9	2.19
		CREAT (µmol/L)	3.25	71.4	13.9	73.5	20.1

				MaHV-6 +ve	MaHV-6 -ve	
			Ct %	Frequency (%)	Frequency (%)	OR ‡ (95% CI)
c.	PBCM (26.16) † +ve n= 12 -ve n= 102	Rouleaux formation	11.0	6 (50)	44 (43.1)	1.32 (0.40-4.37)
		Acanthocytes	11.0	5 (41.7)	53 (52)	0.66 (0.20-2.22)
		Heinz bodies	10.7	5 (41.7)	45 (44.1)	0.90 (0.27-3.04)
		Hypochromasia	9.07	8 (66.7)	85 (83.3)	0.40 (0.11-1.48)
		Schistocytes	6.63	2 (16.7)	23 (22.5)	0.69 (0.14-3.36)
		Echinocytes	6.09	1 (8.3)	24 (23.5)	0.30 (0.04-2.41)
		Flower cells	5.82	2 (16.7)	17 (16.7)	1 (0.2-4.98)
		Keratocytes	5.27	2 (16.7)	14 (13.7)	1.26 (0.25-6.35)
		Anisocytosis	5.24	12 (100)	79 (77.5)	7.39 (0.42-129) *
		nRBCs	5.06	11 (91.7)	86 (84.3)	2.05 (0.25-16.9)
		Poikilocytosis	4.92	11 (91.7)	86 (84.3)	2.05 (0.25-16.9)
		Polychromasia	3.78	12 (100)	86 (84.3)	6.79 (0.39-119) *
		Howell-Jolly bodies	3.54	11 (91.7)	94 (92.2)	0.94 (0.11-8.20)
		Reactive lymphocytes	0.29	12 (100)	101 (99)	0.37 (0.01-9.57) *

Ct: Percent of contribution to difference

† Overall average dissimilarity

‡ Odds Ratio for the presence of the taxon in MaHV-6 PCR positive individuals and 95% confidence intervals calculated using Woolf's method (1955)

* calculated by adding 0.5 to each observed value (Altman 1999)

However, these differences were not significant (ANOSIM: $p>0.05$; Table 6-3), evident in the absence of particular spatial clustering of MaHV-6 positive and negative animals, either compared by sites (nMDS; Figure 6-5a,c,e) or by sexes (nMDS; Figure 6-5b,d,f). There were significant *site* effects for HMT and BLC (ANOSIM: $p> 0.05$; Table 6-3a,b), evident in the clustering of the data points in the 3D models (nMDS analyses: Figure 6-5a,c). By contrast, there was no effect of *site* on PBCM parameters (Table 6-3c). There were no *sex* effects on HMT, BLC, or PBCM variables (Table 6-3a-c).

Table 6-3 Two-way ANOSIM of selected (a) haematology variables (corrected WBC, RBC, HGB, PCV, and absolute counts for leukocytes obtained with a manual differential on a blood smear), (b) blood chemistry analytes (ALP, ALT, AST, CK, PROT, ALB, CALC, PHOSP, CHOL, BILT, GLUC, CREAT, UREA and Vitamin E), and (c) PBCM, for *S. brachyurus* (Rottnest Island and mainland combined sample) that were MaHV-6 PCR-positive and MaHV-6 PCR-negative, with site and sex as independent factors. Bray-Curtis similarity index, Permutation N= 9,999. Only two independent factors could be tested simultaneously, and therefore the presence of MaHV-6 was tested first with site, and then secondly with sex. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

	Variables	Factors	*R*	*p*
a.	HMT	*MaHV-6*	-0.036	0.650
		Site	0.184	**0.015**
		MaHV-6	-0.079	0.735
		Sex	-0.177	0.945
b.	BLC	*MaHV-6*	-0.078	0.809
		Site	0.602	**0.001**
		MaHV-6	-0.081	0.771
		Sex	0.031	0.375
c.	PBCM	*MaHV-6*	-0.107	0.909
		Site	0.076	0.112
		MaHV-6	-0.152	0.915
		Sex	-0.216	0.992

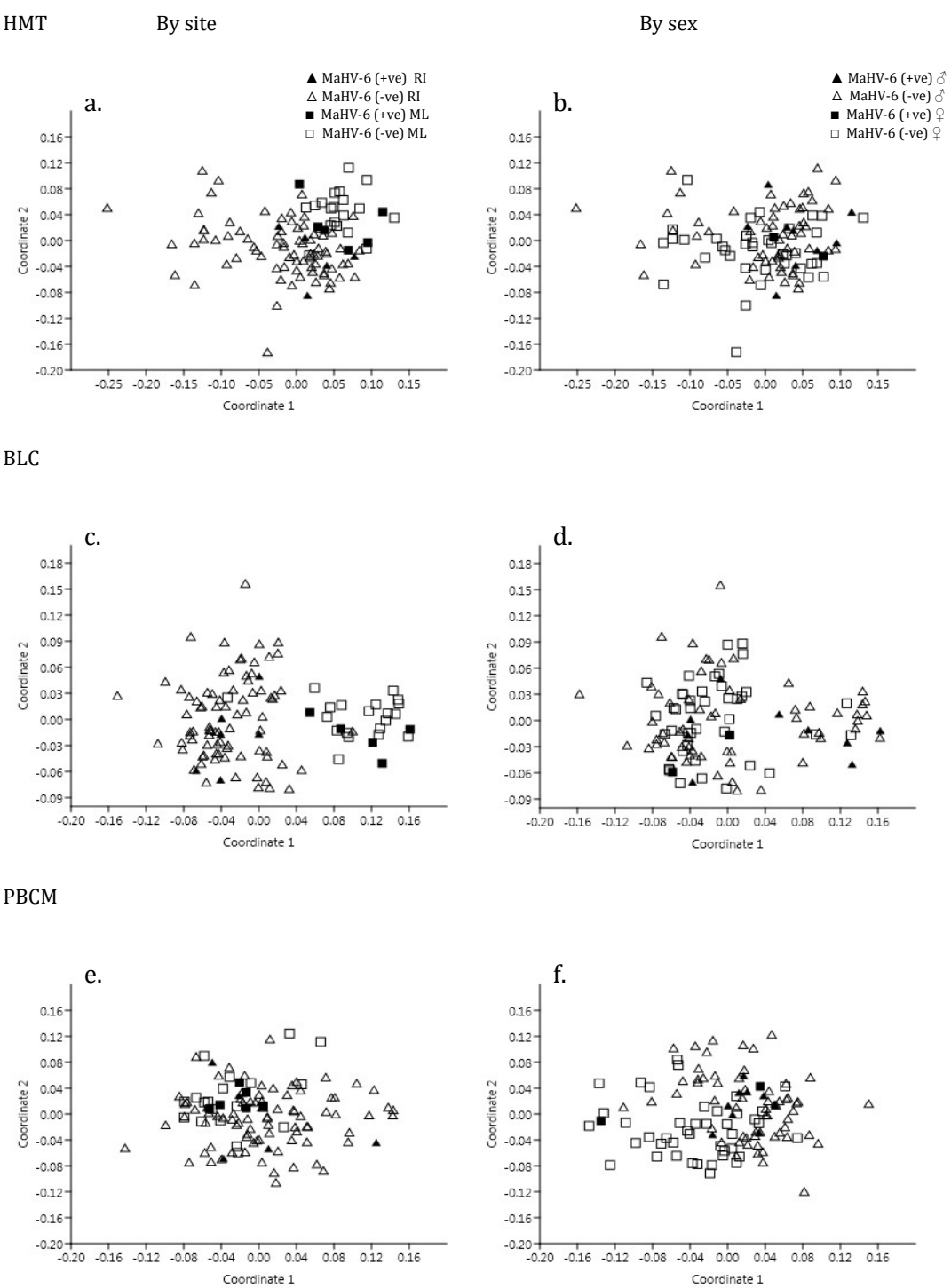


Figure 6-5 Non-metric MDS plots with Bray-Curtis similarity measure, illustrating the structural dissimilarity of HMT (a. stress statistic=0.215; b. stress statistic= 0.175), BLC (c. stress statistic= 0.280; d. stress statistic= 0.236), and PBCM (e, f. stress statistic= 0.28) communities between *S. brachyurus* [Rottnest Island (RI) and mainland (ML) combined sample] that were MaHV-6 PCR-positive (filled symbols) and MaHV-6 PCR-negative (open symbols). Key legends should be read for the left hand and right hand panels separately. Note that the distances along the axes are unit-less, therefore the positions of the points in the plots are relative distances from one another rather than absolute differences read in these units. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

6.4.5 *Relation between MaHV-6 and: (i) physical examination findings, and (ii) other organisms*

Physical examination of all animals tested revealed no associations of clinical signs with the presence of MaHV-6 (Table 6-4). By contrast, there was a significant association between the presence of MaHV-6 and the prevalence of *Theileria* sp. with MaHV-6. This was not the case for co-infection with *Salmonella* spp., microfilariae, *Eimeria* spp., nematode eggs, *Babesia* sp., and trypanosomes (Table 6-5).

Table 6-4 Associations between physical examination findings and MaHV-6 in animals on Rottnest Island and the mainland (combined sample). OR= odds ratio, CI= confidence interval, MM= mucous membranes, EP= external parasites.

Finding	MaHV-6 +ve n (%)$_{(n=13)}$	MaHV-6 -ve n (%)$_{(n=108)}$	OR (95% CI) *	$\chi^2_{1, n=121}$
Abnormal MM [a]	1 (7.69)	23 (21.9)	0.31 (0.04-2.49)	0.63, p= 0.427
Cloudy eye	0 (0)	2 (1.85)	1.58 (0.07-34.6) †	0.43, p= 0.512
Dehydration [b]	5 (38.5)	39 (36.1)	1.11 (0.34-3.61)	0.02, p= 0.890
Ear notches	4 (30.7)	17 (15.7)	2.36 (0.66-8.61)	0.93, p= 0.335
Flaky skin	2 (15.3)	2 (1.85)	9.64 (1.23-75.3)	3.09, p= 0.079
Fractures	1 (7.69)	2 (1.85)	4.42 (0.37-52.4)	0.11, p= 0.737
Fur loss	5 (38.5)	27 (25)	1.88 (0.57-6.22)	0.50, p= 0.480
Presence of EP	6 (46.1)	32 (29.6)	2.04 (0.63-6.53)	1.19, p= 0.276
Skin erosions	4 (30.7)	13 (12)	3.25 (0.87-12.1)	2.00, p= 0.157
Testicular hypoplasia [c]	0 (0)	2 (1.85)	1.58 (0.07-34.6) †	0.43, p= 0.512
Ulcers [d]	1 (7.69)	0 (0)	26 (1.01-674.1) †	1.62, p= 0.203

[a] Pale and blue tinted mucous membranes
[b] Skin tenting for longer than 2 seconds
[c] Decreased size of testes (bilateral or unilateral)
[d] Ulcers found inside the pouch of females
* Odds Ratio for the presence of the taxon in MaHV-6 PCR positive individuals and 95% confidence intervals calculated using Woolf's method (1955)
† calculated by adding 0.5 to each observed value (Altman 1999)

Table 6-5 Yates corrected chi-square results for associations between other infectious agents screened for in *S. brachyurus* (Rottnest Island and mainland combined sample) that were MaHV-6 PCR-positive and MaHV-6 PCR-negative. OR= odds ratio, CI= confidence interval.

	MaHV-6			
Finding	+ve/n (%)	-ve/n (%)	OR (95% CI) †	χ^2
Babesia sp. [a] *	0/13 (0)	1/108 (0.93)	2.65 (0.10-68.4) ‡	$\chi^2_{1,\ n=\ 121} = 1.62$, $p = 0.203$
Cryptococcus spp. [b]	0/8 (0)	5/96 (5.2)	0.98 (0.05-19.2) ‡	$\chi^2_{1,\ n=\ 104} = 0.04$, $p = 0.842$
Eimeria spp. [c]	7/9 (77.7)	51/60 (85)	0.62 (0.11-3.46)	$\chi^2_{1,\ n=\ 69} = 0.00$, $p = 0.949$
Microfilariae [c]	3/13 (23.1)	33/105 (31.4)	0.65 (0.17-2.54)	$\chi^2_{1,\ n=\ 118} = 0.09$, $p = 0.766$
Nematode eggs [c]	8/11 (72.7)	55/59 (93.2)	0.19 (0.04-1.03)	$\chi^2_{1,\ n=\ 70} = 2.35$, $p = 0.125$
Salmonella spp. [d] **	3/5 (60)	23/54 (42.6)	2.02 (0.31-13.1)	$\chi^2_{1,\ n=\ 59} = 0.08$, $p = 0.780$
Theileria sp. [a]	11/13 (84.6)	36/108 (33.3)	**11.0 (2.31-52.3)**	$\chi^2_{1,\ n=\ 121} = 10.8$, $\mathbf{p = 0.001}$
Trypanosomes [a] ***	1/13 (7.7)	2/108 (1.85)	4.42 (0.37-52.4)	$\chi^2_{1,\ n=\ 121} = 0.113$, $p = 0.737$

[a] Screened by PCR (piroplasms and trypanosomes: Chapter 7, section 7.3.1.1]
[b] Screened by culture methods, species and varieties determined by PCR (see Chapter 5, section 5.3.1)
[c] Screened by light microscopy [gastrointestinal parasites: see Chapter 3, section 3.4.1; microfilariae: see Chapter 7, section 7.3.1]
[d] Screened by culture methods, species and serovars determined by serotyping (see Chapter 4, section 4.2.1)
* positive animal from Rottnest Island cohort
** Rottnest Island sample only
*** all positive animals from mainland cohort
† Odds Ratio for the presence of the taxon in MaHV-6 PCR positive individuals and 95% confidence intervals calculated using Woolf's method (1955)
‡ calculated by adding 0.5 to each observed value (Altman 1999)

6.5 Discussion

Free-ranging *S. brachyurus* were nPCR-positive for a novel gammaherpesvirus (proposed to be designated as MaHV-6) when tested by a pan-herpesviral nested PCR of peripheral blood. The virus was present in 10.7% of 121 individuals sampled across both Rottnest Island and mainland WA populations, with a greater prevalence on the mainland (25%) compared to Rottnest Island (6.45%) (p= 0.011). Even though the relative risk of infection was 3.72 times higher for males than females, this was not statistically significant (p= 0.077). There was also no influence of season on the prevalence of this virus. Multivariate analyses showed that MaHV-6 had no effect on the HMT, BLC or PBCM, and there was no significant association between the presence of MaHV-6 and physical examination findings. There was a significant association between the presence of MaHV-6 and coinfection with *Theileria* sp. (p=0.001; detected in peripheral blood). This may suggest that quokkas may be more susceptible to infection with *Theileria* sp., if infected with MaHV-6.

The DPOL gene is a highly conserved region of the viral genome and is one of the more commonly sequenced herpesviral genes. These features make the DPOL gene well suited to genetic comparisons of herpesvirus species (Pellet & Roizman 2007). The predicted amino acid sequence obtained for MaHV-6, is consistent with this virus being a novel member of the *Gammaherpesvirinae* subfamily. Our alignment highlights substantial

differences in the predicted amino acid sequence (when compared to the most homologous sequence) of a highly conserved region of the DPOL gene. VanDevanter et al. (1996) observed only single nucleotide base variations in the DPOL gene between strains within a single virus species without repercussion in the amino acid sequence (i.e. 17 strains of human herpesvirus 6, five strains of human herpesvirus 2, and five strains of human herpesvirus 7). The gammaherpesvirus described in this paper shared a 76% identity in the amino acid sequence (42/55 amino acids) with the most homologous known macropodid herpesvirus, MaHV-5. Pairwise identity was lower when aligned with other recently characterised marsupial gammaherpesviruses (i.e. 73% MaHV-3, 56% PhaHV-1, 52% PhaHV-2, 56% DaHV-1 and 47% PotHV-1). Similar or higher percentages of sequence identity to the most homologous sequence have been obtained in other studies where the newly detected herpesvirus amino acid sequence has been proposed as new species. For instance, Tortoise HV-2 (83% sequence identity with that of Tortoise HV-1) (Johnson *et al.* 2005), *Mustelid herpesvirus* 2 (91% sequence identity with that of *Mustelid herpesvirus* 1) (Tseng *et al.* 2012), Beluga whale herpesvirus (79.5% sequence identity with bovine alphaherpesvirus 5) (Bellehumeur *et al.* 2015), and *Lynx rufus gammaherpesvirus* 1 (79% sequence identity with that of *Mustelid herpesvirus* 1) (Troyer *et al.* 2014). Following current conventions in herpesviral nomenclature, we have tentatively designated the unknown virus as Macropodid herpesvirus-6 (MaHV-6). Our results place MaHV-6 within *Gammaherpesvirinae*; however, the distance and location of this virus in the dendrogram (Figure 6-2) should be seen as approximate, and more studies would be necessary to further characterise the relation between MaHV-6 and other gammaherpesviruses.

Although a longer sequence would have provided more information for sequence alignment and consequently a greater resolution to our phylogenetic analyses (Flynn *et al.* 2005), attempts to increase the length of the nPCR product obtained, and to amplify other viral genomic regions of MaHV-6 by designing new primers using reported sequences of macropodid herpesviruses, were unsuccessful (data not shown). Despite the relatively short length of our amino acid sequence, we regard this number of substitutions to be consistent with the amount of variability in the nucleotide residues that would be attributable to a related, but genetically distinct, novel species of herpesvirus. Although a false positive result (i.e. the detection of herpesviral DNA in peripheral blood when there is no herpesviral DNA) may be possible, it is highly unlikely. The set of primers used in this study has been shown to not generate spurious PCR products even in the absence of appropriate template (VanDevanter *et al.* 1996). Furthermore, there is no amplification of polymerase genes from other organisms DNA, but herpesvirus DNA only, given that the

coding region of the polymerase gene targeted by these primers is unique to herpesvirus species (VanDevanter *et al.* 1996). Additionally, safety mechanisms were put in place to enhance the reliability of the results (i.e. positive control and negative controls).

To our knowledge, MaHV-6 constitutes the third gammaherpesvirus detected in macropods, and the first identification of a member of *Gammaherpesvirinae* in animals across multiple subpopulations of free-ranging and apparently healthy macropods in WA. The animals examined in this study showed no obvious signs of clinical disease resembling herpesviral infection in macropods (e.g. cloacal and peri-cloacal ulcerations, pyrexia, pneumonia, conjunctivitis, rhinitis, tail twitching, and nasal and ocular discharges; Acland 1981 ; Finnie, Littlejohns, & Acland 1976 ; Smith, Wellehan Jr*, et al.* 2008 ; Vaz *et al.* 2013 ; Wilcox *et al.* 2011 ; Wilks, Kefford, & Callinan 1981). Of the previously reported macropodid herpesviruses, all except one (MaHV-5; Stalder *et al.* 2015) have been detected in diseased individuals; this makes our finding epidemiologically relevant. MaHV-1 and MaHV-2 (both alphaherpesviruses) have been linked to disease in captive macropods, MaHV-3 has been linked with disease in both captive and free-ranging eastern grey kangaroos, while MaHV-4, also in eastern grey kangaroos, has been linked to disease under free-ranging conditions (Table 6-6).

A range of evidence strongly suggests that the quokka is the natural host for MaHV-6. Firstly, we found no association with MaHV-6 and obvious signs of disease typically associated with herpesviral infection, which may be typical for a herpesvirus with a long association with that host. Secondly, identical herpesviral amino acid sequences were detected in free-ranging *S. brachyurus* from mainland Australia as well as Rottnest Island (which separated from the mainland approximately 7,000 years ago due to raising waters; Glenister, Hassell, & Kneebone 1959), suggesting that the virus was present in the population before the island population became isolated from the mainland population. Thirdly, members of *Gammaherpesvirinae* usually have a narrow host range (MacLachlan & Dubovi 2011) and are thought to have coevolved with their host species over millions of years (Davison 2002). We therefore believe that the quokka is the natural host for MaHV-6.

Unlike members of *Alphaherpesvirinae* and *Betaherpesvirinae* subfamilies, gammaherpesviruses are particularly specific for either B- or T- lymphocytes (Markey *et al.* 2013 pp. 559-573) and have the tendency to avoid lytic replication and favour latent infections instead (Ackermann 2006). As a result, the infection persists in the infected host with little or no virus replication and gene expression (Speck & Ganem 2010). This

may have been the case in our study, where the animals we screened may have been infected with MaHV-6 but the little or no replication of the same may have compromised DNA recovery. Furthermore, none of the animals in our sample had obvious clinical signs of disease compatible with any of the other known macropodid herpesviruses. With the exception of detection of gammaherpesviruses in apparently healthy swamp wallabies (*Wallabia bicolor*) (Stalder 2013), and in apparently healthy eastern bettongs (*Bettongia gaimardi*) (Portas *et al.* 2014), all other studies of herpesviruses in marsupials have detected the viruses by PCR from diseased animals. Furthermore, with the exception of the study by Smith et al. (2008; who also screened whole blood), previous studies have screened tissue samples (e.g. liver, lung, kidney and spleen), or swabs (e.g. conjunctiva, cloaca, prepuce and nasal lining) (e.g. Portas *et al.* 2014 ; Stalder 2013). However, of 17 animals found positive to MaHV-3 by nPCR (tissue swabs and biopsies), the virus was only detected in three animals when blood was screened (Smith, Wellehan Jr*, et al.* 2008). Therefore, MaHV-6 may be more prevalent than we recorded, which may be found by screening tissue, conjunctiva, and nasal lining samples (HVs are likely to be shed from this site during recrudescence; Hüssy *et al.* 2002), especially from diseased or dead animals.

Generally, animals in this study were apparently healthy. Although one female presented with ulcers in its pouch and another individual presented conjunctivitis, statistical analyses determined no association of these with the presence of MaHV-6. Furthermore, we did not detect an effect of the presence of MaHV-6 on the HMT, BLC, or PBCM. Our findings agree with previous studies in macropods (Stalder 2013), other marsupials (Portas *et al.* 2014), as well as with studies in other host species (Bicknese, Childress, & Wellehan Jr 2010 ; Mugisha *et al.* 2010 ; Stanton *et al.* 2014) showing that herpesviruses can be carried asymptomatically. By contrast with the finding of MaHV-6 presence or not, site had an evident effect on the HMT and BLC of Rottnest Island and mainland animals, which could be attributed to the interplay of multiple factors, including nutrition, social structure, age, behaviour, underlying disease and genetic differences.

Table 6-6 Summary of the *Macropodid* and other marsupial herpesviruses isolated to date. Subf.= subfamily, NC= not characterised, DaHV= dasyurid herpesvirus, MaHV= macropodid herpesvirus, PhaHV= phascolarctid herpesvirus, PotHV= potoroid herpesvirus, VoHV= vombatid herpesvirus. *Alphaherpesvirinae* (α), *Betaherpesvirinae* (β), and *Gammaherpesvirinae* (γ).

Herpesvirus	Subf.	Host species	Habitat	Clinical signs	Reference
DaHV-1	γ	Yellow-footed antechinus (*Antechinus flavipes*) Agile antechinus (*A. agilis*)	Free-ranging (South Australia)	Weakness, poor body condition	(Amery-Gale *et al.* 2014)
DaHV-2	γ	Tasmanian devil (*Sarcophilus harrisii*)	Free-ranging	Asymptomatic	(Stalder *et al.* 2015)
MaHV-1	α	Parma wallaby (*Macropus parma*)	Captive	Respiratory signs, conjunctivitis, incoordination, pyrexia, as well as cloacal/pericloacal ulcers	(Acland 1981 ; Finnie, Littlejohns, & Acland 1976 ; Johnson & Whalley 1990)
MaHV-2	α	Dorcopsis wallaby (*Dorcopsis muelleri luctuosa*) Quokka (*Setonix brachyurus*)	Captive - mixed-species enclosure (Melbourne Zoo)	Oral ulcers, persistent bilateral purulent conjunctival exudate; quokka found dead 24 h after clinical signs noted	(Callinan & Kefford 1981 ; Johnson & Whalley 1987 ; Johnson *et al.* 1985 ; Wilks, Kefford, & Callinan 1981)
MaHV-3	γ	Eastern grey kangaroo (*M. giganteus*)	Captive (Fort Wayne Children's Zoo, USA)	Ulcerative cloacitis, mild cloacal discharge, overgrooming, mammary masses; death	(Smith, Wellehan Jr, *et al.* 2008)
			Free-ranging (eastern Australia)	Nasal and bilateral serous ocular discharges, pyrexia, respiratory disease; dead individuals in the mob	(Wilcox *et al.* 2011)
MaHV-4	α	Eastern grey kangaroo (*M. giganteus*)	Free-ranging	Respiratory, ocular and possibly nervous system disease	(Vaz *et al.* 2013).
MaHV-5	γ	Swamp wallaby (*Wallabia bicolor*)	Free-ranging	Asymptomatic	(Stalder 2013)
MaHV-6	γ	Quokka (*S. brachyurus*)	Free-ranging	Asymptomatic	Present study
PhaHV-1 PhaHV-2	γ	Koala (*Phascolarctos cinereous*)	Free-ranging (eastern Australia)	Weakness, conjunctivitis	(Vaz *et al.* 2012 ; Vaz *et al.* 2011)
PotHV-1	γ	Eastern bettong (*Bettongia gaimardi*)	Free-ranging (Tasmania)	Asymptomatic	(Portas *et al.* 2014)
VoHV-1 VoHV-2	γ	Common wombat (*Vombatus ursinus*)	Free-ranging	Asymptomatic	(Stalder *et al.* 2015)
NC		Red kangaroo (*M. rufus*)	Captive (private animal dealer, USA)	Progressive 1-wk illness with no specific signs of disease	(Britt Jr, Frost, & Cockrill 1994)
		Brush-tailed rock wallaby (*Petrogale penicillata*), Dusky pademelon (*Thylogale brunii*)	Captive (wildlife reserves, zoos and wildlife carers)	Herpesvirus infection was documented on histological sections of liver lesions held at the Australian Registry of Wildlife Health (ARWH)	(Canfield & Hartley 1992)

Many herpesviruses are persistent life-long infections with limited but periodic virus replication (viraemia) (MacLachlan & Dubovi 2011) that leads to virus shedding and transmission. Reactivation may occur (even in host-adapted herpesviruses) as a result of immunosuppression from concomitant disease or stress, leading either to shedding without signs of disease or to clinical disease in its natural host (Barrandeguy *et al.* 2008 ; Ledbetter *et al.* 2012 ; Rock *et al.* 1992 ; Roizman & Pellet 2001). This reactivation was observed in apparently healthy *M. giganteus* (seropositive to MaHV-1) that resulted shedding an alphaherpesvirus similar to MaHV-1 and MaHV-2, after being treated with dexamethasone (a glucocorticoid capable of immunosuppression) (Guliani *et al.* 1999).. Increased endogenous glucocorticoids observed during stressful conditions, can similarly result in reactivation of herpesviral infections (Sykes 2014 ; Tanaka & Mannen 2003 ; Winkler *et al.* 2002). Immunosuppression of MaHV-6 infected quokkas could occur under the pressure of external factors (e.g. temperature and nutritional challenge, habitat destruction, or predation threat), as well as internal factors (e.g. concomitant infection), which in turn could contribute to parasite-optimal conditions that may lead to shedding, disease and possibly fatal disease in *S. brachyurus* (Harvell *et al.* 2002 ; Pellet & Roizman 2007). It is not known what the association ($p<0.05$) between MaHV-6 with *Theileria* sp. signify for the quokka, thus further studies are necessary to determine the factors responsible for such, as well as the impacts of these co-infections on the health of quokkas. The absence of clinical signs in the animals tested therefore does not imply that animals would be free of herpesviral disease under all conditions.

In common with other more completely studied herpesviruses, it is likely that MaHV-6 may be a persistent life-long infection with limited but periodic virus replication (MacLachlan & Dubovi 2011). If this is the case, this viral replication may lead to shedding and transmission. In turn, *S. brachyurus* on the mainland and on Rottnest Island may consequently be a reservoir of MaHV-6 that could spill over to other species sharing their habitat; a situation that may carry potentially hazardous outcomes for both captive and free-ranging populations, considering that herpesviral infections are usually fatal for species different from the natural host.

From a conservation perspective, fragmented quokka populations may be at greater risk of disease due to changing environmental conditions, which have been suggested to be linked to an increase in pathogen transmission, and a decrease in survival rates and host susceptibility (Gibson *et al.* 2010 ; Harvell *et al.* 2002 ; Pinto *et al.* 2008). A better understanding of this virus in *S. brachyurus* could allow for a quicker response if clinical disease were to occur. Our findings provide baseline data that can be used in *ex-situ* and

in-situ conservation programs of *S. brachyurus*, and contribute to the current knowledge of herpesviruses in Australian marsupials. Future studies should aim to isolate in cell culture and further characterise this virus as well as establish the extent of distribution of MaHV-6 in free-ranging *S. brachyurus* by including individuals from other sub-populations. Similarly, further work should also aim to investigate the presence of circulating neutralising antibodies to this gammaherpesvirus in free-ranging related species, and determine transmission, infectivity and overall clinical significance of this virus in the quokka.

7. Haematology and blood chemistry of free-ranging quokkas (*Setonix brachyurus*) from Rottnest Island and selected locations on the mainland of Western Australia

STATEMENT OF AUTHOR CONTRIBUTION

P. Martinez-Perez: designed the general scope and structure of the chapter, initiated the research, carried out all fieldwork procedures, carried out blood smear assessment (i.e. differential counts, erythrocyte morphologies, detection of haemoparasites), entered and analysed the data, and wrote the chapter.

P.A. Fleming: advised and assisted with statistical analyses, and provided editorial comments to versions of the chapter from draft to final version.

T.H. Hyndman: provided editorial comments to versions of the chapter from draft to final version.

A. Paparini: carried out the molecular detection of piroplasms and trypanosomes.

Murdoch University Veterinary Hospital: carried out haematology (except blood smear assessment) and blood chemistry measurements.

DAFWA-Animal Health Laboratories, Western Australia: carried out vitamin E determinations.

7.1 Abstract

Blood was collected from 149 wild quokkas (*S. brachyurus*) from Rottnest Island and mainland Western Australia (WA), between September 2010 and December 2011, to establish haematology (HMT) and blood chemistry (BLC) reference intervals, and test for piroplasms and trypanosomes by nPCR. Differences between subpopulations, seasons and sexes were investigated. Haematology, BLC and peripheral blood cell morphologies (PBCM) data, vary significantly between Rottnest Island and the mainland groups. Rottnest Island animals had lower red blood cell concentration, packed cell volume, and haemoglobin, along with marked evidence of oxidative injury and bone marrow response.

This was consistent with a regenerative normocytic hypochromic anaemia. By contrast, except alkaline phosphatase, all blood chemistry analytes where higher in mainland animals, with particular emphasis on creatine kinase, alanine amino transferase, aspartate amino transferase and vitamin E. Generally, HMT and BLC differences were attributed for the most part to the interplay of multiple factors (e.g. diet, habitat). However, stress and capture myopathy in the mainland group may had played a role in the values obtained. Captive animals may be deficient in vitamin E given that their island conspecifics had plasma values significantly higher (between two to five times). There was no significant difference in the prevalence of microfilariae of filarial nematodes in mainland and Rottnest Island groups. *Theileria* sp. and *Babesia* sp. were detected by nested polymerase chain reaction (nPCR) in Rottnest Island animals, while *Theileria* sp. was the only piroplasm detected in the mainland group. Atypical lymphocytes resembling those in proliferative disorders of the lymphoid and haematopoietic tissues in other species were observed in blood smears of Rottnest Island animals but not in mainland animals. Our data provides the first BLC reference intervals for *S. brachyurus*, and constitutes the most comprehensive haematological analysis for the species. More important, these baseline data represents a tool that would enable health and disease surveillance of free-ranging individuals, activities required for the persistence of the species.

7.2 Introduction

The quokka is a small, diprotodont wallaby marsupial endemic to WA (Kitchener 1995), and is the only member of the genus *Setonix* in the Macropodidae family (Sharman 1961). Population estimates suggest that there are fewer than 18,000 animals across all populations (see Chapter 2, section 2.3.2), however, the elusive nature of this marsupial on the mainland and the limitations of population census techniques reduce the accuracy of this figure. The quokka is currently listed as "fauna which is rare or likely to become extinct" under the Western Australian Wildlife Conservation Act 1950 Section 14(2) (ba); and as "threatened fauna" subcategory "vulnerable" under the Commonwealth of Australia's Environment Protection and Biodiversity Conservation Act 1999. At an international level, *S. brachyurus* holds a conservation status classification of "vulnerable" according to the International Union for Conservation of Nature (IUCN) (de Tores *et al.* 2008).

Studying HMT and BLC parameters is important because they serve as an indicator assessment of an animal's health. These analytes may confirm presumptive diagnoses

based on physical examination observations, establish the extent of impact of a disease, or determine response to therapy; however, the usability of these test results can be jeopardised by the absence of baseline data against which the degree of change can be assessed. According to Bennett et al. (2007), interpretation of haematological studies of western barred bandicoots (*Perameles bougainville*) with papillomatosis and carcinomatosis syndrome (as well as apparently healthy individuals) was hindered by the absence of reference intervals. Furthermore, prompt and educated responses in the event of a disease outbreak will be facilitated by the existence of data on the HMT and BLC values of apparently healthy animals of a particular species. Changes in haematology and blood chemistry responses can indicate underlying disease, stress, nutritional deficiencies and other conditions (Harvey 2012). Accordingly, baseline haematology and blood chemistry reference intervals in conjunction with other tools could be an effective management tool in evaluating the health status of wild populations (McKenzie, Deane, & Burnett 2002). However, reported reference intervals of HMT and BLC analytes are frequently obtained from small populations or populations in captivity (Rostal *et al.* 2012).

Because of their vulnerability to a broad range of stressors (anthropogenic and natural), understanding the HMT and BLC responses of quokkas may aid in the management of free ranging individuals. There have been only two studies of free-ranging quokka, and studies of Rottnest Island animals are generally for captive and semi-captive conditions and provide limited data (Table 7-1). Of these previous studies, only three sources reported white blood cell data (i.e. white blood cell count, differential counts) (Hawkey 1975 ; Lewis, Phillips, & Hann 1968 ; Vogelnest & Portas 2010) and only one source reports biochemical data for *S. brachyurus* (Teare 2002). In addition to the scarce data available for animals on the mainland of WA, small sample size has generally been a limiting factor (Table 7-1). This issue is also present in the HMT and BLC data reported by the International Species Information System (ISIS), with sample sizes ranging between two and four animals per analyte (Teare 2002). In this study, we examined the differences in the HMT and BLC profiles of more than 100 free-ranging *S. brachyurus* on Rottnest Island and on the mainland of WA (Jarrahdale, Collie and Walpole locations), and for the Rottnest Island subsample, we explored the possible influence of season and sex. We also constructed reference intervals for selected HMT and BLC parameters in apparently healthy and free-ranging *S. brachyurus* on both locations (Rottnest Island and the mainland), using statistical methods in line with the C28-A3 guidelines of the International Federation of Clinical Chemistry (IFCC) and Clinical and Laboratory Standards Institute (CLSI) (CLSI 2008).

Table 7-1 List of available data for haematology (H) and blood biochemistry (B) parameters for the quokka *Setonix brachyurus*. C= captive, FR= free-ranging.

Animals sourced from	Condition	Sample size	H	B	Reference
Mainland [1]	FR	5	yes	no	(Clark & Spencer 2006)
Bald Island [2]	FR	7	yes	no	(Clark & Spencer 2006)
Rottnest Island	unknown	10	yes	no	(Clark 2006)
Rottnest Island	FR	38	yes	no	(Shield 1971)
Rottnest Island	C	8-12	yes	no	(Barker 1961)
Rottnest island	C	11	yes	yes †	(Barker *et al.* 1974)
Rottnest Island	C	1	yes ‡	no	(Lewis, Phillips, & Hann 1968)
Rottnest Island	C	43	yes §	no	(Kaldor & Morgan 1986)
Rottnest Island	C	1	yes ‡	no	(Hawkey 1975)
Rottnest Island	C [3]	5-43	yes ‡	no	(Vogelnest & Portas 2010)
Rottnest Island	C	2-4	yes ‡	yes	(Teare 2002)

[1] Two locations on the mainland near Albany region: ~418 km SE of Perth
[2] Bald Island (east of Albany).
[3] Taronga Zoo, Sydney, Australia
† Protein concentrations (total protein, albumin, beta globulin, gamma globulin, alpha globulins 1 and 2, plasma urea)
‡ White blood cell count and differential counts only
§ Haemoglobin only

7.3 Materials and methods

Between September 2010 and December 2011, 153 *S. brachyurus* were captured from different subpopulations stretching across the species' current natural range (see Chapter 2, section 2.3.1). These sites included Rottnest Island and several locations on the mainland of WA (i.e. Jarrahdale, Collie and Walpole) (see Chapter 3, sections 3.1.1 and 3.1.2). Trapping methods differed between island and mainland sites with Thomas traps® (Sheffield Wire Products, WA Australia) used on Rottnest Island (hand capture was also used) and the mainland southern sites (i.e. Walpole), and Sheffield traps® (Sheffield Wire Products, Sheffield Rd Welshpool, WA Australia) used on the mainland northern sites (i.e. Jarrahdale and Collie). On Rottnest Island, traps were deployed and cleared out every hour during the sampling session, which meant that animals spent no more than 1 hour in the trap before being removed. On the mainland, traps were deployed and left open overnight, which meant that animals could have been in the trap for several hours before being processed. Physical examination and sample collection were performed while under general anaesthesia (see Chapter 3, section 3.3). Blood was successfully obtained from 149 individuals; reference intervals and multivariate analyses were calculated on different sample sizes due to unforseen issues with analyses (e.g. insufficient blood sample volume, haemolysis and blood smears not having adequate cell distribution and quality). Additionally, blood samples from eight captive adult and apparently healthy quokkas that for the most part were handled under similar protocols (i.e. trapping, anaesthesia), were

collected in winter by Perth Zoo personnel and kindly given to this project for vitamin (vit.) E analyses. As of March 2015, the diet provided to quokkas at Perth Zoo, included macropod pellets (unknown concentration of vit. E), sprinkled with Value Plus Vitamin E Powder ® (Value Plus Animal Health Care Products Pty Ltd, NSW Australia) at a dose of 1 g/animal/day (i.e. 52 mg of d-alpha tocopheryl acid succinate); quokka cubes (Specialty Feeds, WA Australia) containing 1,600 mg of alpha tocopherol acetate per 1 kg of product; Olsson's 007 Pressed Nutritional Mineral Block ® (Olsson's, WA Australia) (vit. E concentration not available); browse species (e.g. *Acacia*, *Brachychiton*, and *Ficus*), and lucerne hay (also know known as Alfalfa).

Blood samples for HMT and BLC (Rottnest Island, mainland WA, and Perth Zoo) were collected from the lateral left or right tail veins. Skin was prepared by clipping the venipuncture site and disinfecting with a mixture of chlorhexidine gluconate and 70% ethanol solution at a 1:1 ratio. Blood was obtained using Safety-Lok™ BD Vacutainer® with either a 25G x ¾" or 23G x ¾" needle with Slip Tip 3 mL syringes (Becton, Dickinson and Company, NJ, USA). Blood for blood chemistry (~1000 µL) analyses were collected in 1.3 mL Micro Tubes with 35 I.U. of lithium heparin (SARSTEDT, Aktiengesellschaft & Co. Nümbrecht, Germany), while samples for haematology analyses (~500 µL) were collected in 600 µL BD Microtainer® tubes with potassium (K_2) ethylene diamine tetraacetic acid (EDTA) anticoagulant (Becton, Dickinson and Company, NJ, USA). These two blood samples were mixed gently upon collection and stored at 4 ˚C for further processing. Peripheral blood smears were made using the spreader slide technique and air-dried for storage.

7.3.1 *Haematology and Blood chemistry*

Blood in EDTA and Lithium Heparin vials was submitted to the Clinical Pathology service of the Murdoch University Veterinary Hospital within 96 h of collection or less, for a complete blood count and BLC analyses. Except vitamin E, all BLC analytes were measured in an RX Daytona™ automatic biochemistry analyser (Randox Laboratories). Plasma samples were sent to the Animal Health Laboratories (AHL) at Department of Agriculture and Food of Western Australia (DAFWA) for vitamin E analyses using the method of McMurray and Blanchflower (1979). The chromatographic separation was performed with an Agilent HPLC system (1100) on a Zorbax SB-C18 column (3 mm x 150 mm, 3.5 µm) (Agilent Technologies) with a methanol mobile phase. Alpha-tocopherol (vitamin E) was quantified using fluorescence detection (ex. 296 nm and em. 330 nm). A complete blood count and some erythrocyte indices were obtained with an ADVIA-120®

automated haematology analyser (Bayer diagnostics division, Tarrytown, New York, USA) and multi-species software using the default setting (canine). We report Corpuscular Haemoglobin Concentration Mean (CHCM) instead of Mean Corpuscular Haemoglobin Concentration (MCHC), given that the former is obtained through laser-detection technology that allows for direct determination of haemoglobin in each erythrocyte, therefore not being affected by blood abnormalities that could increase the spectrophotometric reading (e.g. lipaemia, haemolysis) (Thrall 2012a) which would be the case of MCHC (calculated).

Considering that light scattering and impedance (i.e. volumetric sizing) standards do not exist for *S. brachyurus*, and that the algorithms of the ADVIA-120® cannot adjust to morphological variations of the cells of interest, differential leukocyte counts obtained through the ADVIA-120® automated haematology analyser were considered unreliable. Consequently, results obtained through the manual differential leukocyte count, a technique that has been used in recent studies as a reference standard (Briggs *et al.* 2009 ; Meintker *et al.* 2013 ; Welles, Hall, & Carpenter 2009), were used to construct reference intervals and in multivariate analyses. The ADVIA-120® nucleated blood cell count was corrected by subtracting the polychromatophilic erythrocytes count. This corrected white blood cell count was then used in subsequent analyses. Variables measured and used in all analyses are presented in Table 7-2.

Blood smears were stained with a Hema-tek® Slide Stainer using Hema-tek® Wright's Giemsa stain (Ames Company, Miles Laboratories). Smears were then assessed using light microscopy to determine the differential leukocyte count (200 leukocytes) and polychromatophilic erythrocyte count (1,000 erythrocytes) at x400 magnification. The in-house interpretation of neutrophils, eosinophils, basophils, lymphocytes and monocytes (Figure 7-1), was based on comparison with published information for these cell populations in *S. brachyurus* (Clark 2004). Additionally, erythrocyte morphology was assessed, including poikilocytes (i.e. atypically shaped erythrocytes) such as echinocytes, keratocytes and spherocytes; structures inside the erythrocyte (e.g. Heinz bodies and Howell-Jolly bodies) and atypical erythrocyte arrangement (e.g. Rouleaux formation) (Figure 7-1). Atypical leukocytes were also recorded (e.g. toxic changes in neutrophils: Döhle bodies), along with free-circulating parasitic organisms (i.e. microfilariae and trypanosomes). Abnormal cell morphologies, such as Döhle bodies and Heinz bodies, were assessed during the 200 leukocyte differential count at x1000 magnification. Presence and numbers of microfilariae were obtained by examining the complete blood smear at x40 magnification but anatomical characteristics of microfilariae were recorded

at x400 or x1000 magnification. The presence of intraerythrocytic organisms was assessed by examining 100 fields within the monolayer and feathered regions of the blood smear combined, first at x400 magnification, and subsequently at x1000 magnification. ImageJ v1.49t (Schneider, Rasband, & Eliceiri 2012) was used to make life measurements of microfilariae and piroplasms. Prevalence of piroplasms and trypanosomes was finally determined by nPCR methods.

Table 7-2 List of haematology and blood chemistry analytes measured on whole blood and plasma obtained from mainland and Rottnest Island *S. brachyurus*. Vitamin E was also measured in nine captive *S. brachyurus* from Perth Zoo.

Analyte	Acronym	Sample type
Haematology		
White Blood Cell Count	WBC	Whole blood in EDTA
Red Blood Cell Concentration	RBC	
Haemoglobin Concentration	HGB	
Packed Cell Volume	PCV	
Mean Corpuscular Volume	MCV	
Corpuscular Haemoglobin Concentration Mean	CHCM	
Red Cell Distribution Width	RDW	
Platelet Concentration	PLT	
Neutrophils	NEUT	
Lymphocytes	LYMPH	
Monocytes	MONO	
Eosinophils	EOS	
Basophils	BASO	
Blood Chemistry		
Alkaline Phosphatase	ALP	Whole blood in Lithium-Heparin
Alanine Aminotransferase	ALT	
Aspartate Aminotransferase	AST	
Creatine Kinase	CK	
Gamma-glutamyl Transferase †	GGT	
Total Protein	TP	
Albumin	ALB	
Globulin	GLOB	
Calcium	CALC	
Phosphorus	PHOSP	
Cholesterol	CHOL	
Total Bilirubin	BILT	
Glucose	GLUC	
Creatinine	CREAT	
Urea	UREA	
Vitamin E	Vit. E	

† included only for reference intervals due to sample size restrictions for multivariate analyses

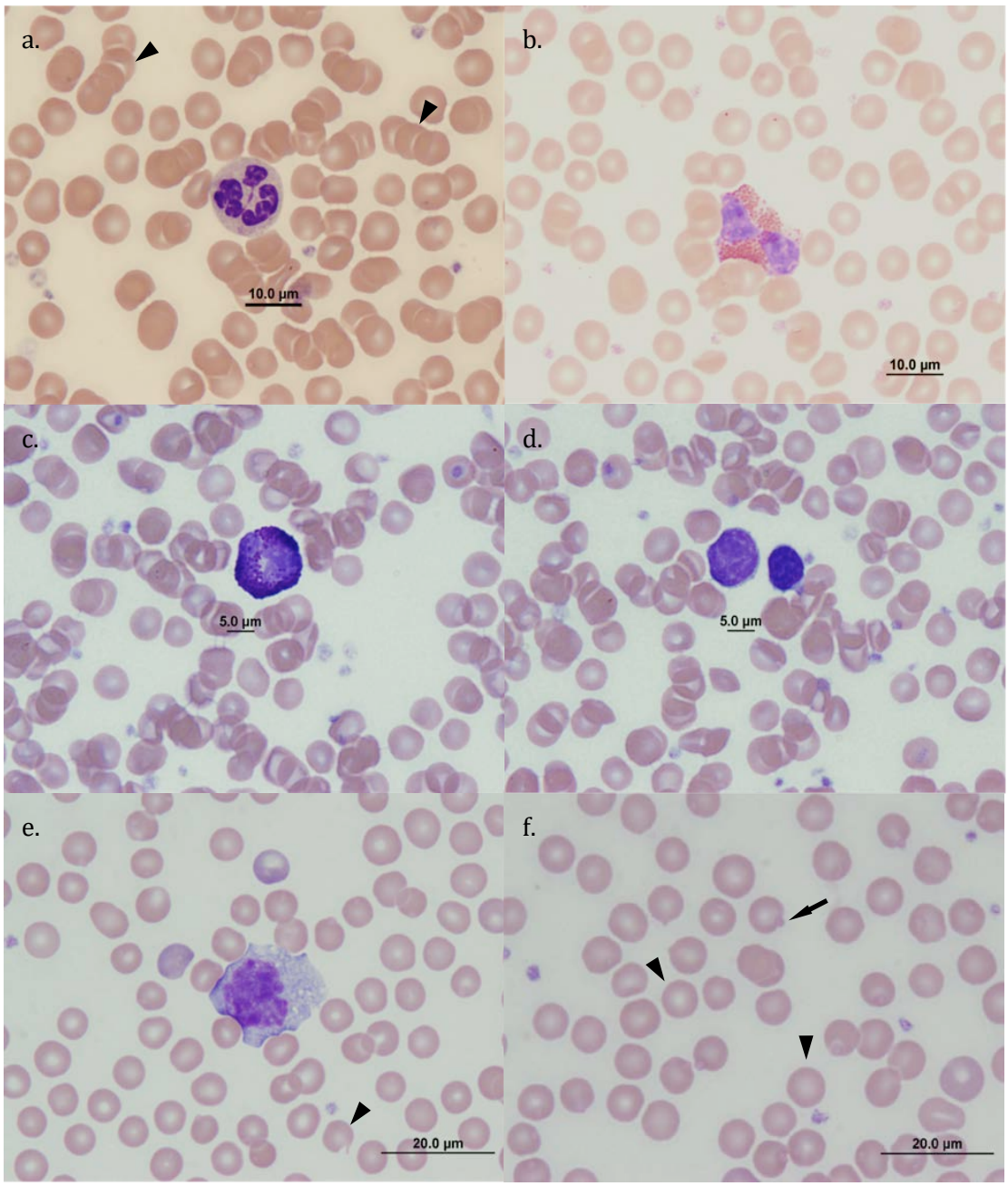


Figure 7-1 Photomicrographs of reference leukocytes and red blood cells observed in blood smears of *S. brachyurus* on Rottnest Island and the mainland of Western Australia. (a) neutrophil, key features: polylobated nucleus with 3-6 lobes, coarsely clumped chromatin, primary cytoplasmic granules -azurophilic-; also present, atypical arrangement of erythrocytes: Rouleaux formation (arrowheads); (b) eosinophil, key features: polylobated nucleus with 2-3 lobes, coarsely clumped chromatin but less dense than neutrophils, prominent secondary eosinophilic cytoplasmic granules; (c) basophil, key features: intense secondary basophilic cytoplasmic granules; (d) lymphocytes, key features: darkly staining chromatin with no apparent nucleolus, cytoplasm presents as a "rim" and appears finely or coarsely granular, nuclear:cytoplasm (N:C) ratio smaller than that of other leukocytes; (e) monocyte, key features: indented to irregularly shaped nucleus, reticular chromatin, large amount of pale, grey to basophilic cytoplasm, often with vacuoles, overall size of the cell is greater than all other leukocytes; also present a keratocyte (i.e. poikilocyte; arrowhead) (f) erythrocytes with normal morphology (arrowheads); RBC inclusion (e.g. Heinz body -arrow-). All photomicrographs: original magnification x1000-, staining Wright-Giemsa.

7.3.1.1 Molecular detection of piroplasms and trypanosomes

Genomic DNA was extracted from 25 µL to 100 µL of whole blood in EDTA, using ISOLATE II Blood DNA Kit (Bioline Reagents Ltd., London, United Kingdom) according to the manufacturer's instructions (see Appendix 10.4.1). Extractions were also made from sterile molecular-grade water as a negative control. A total of 121 animals were screened for piroplasms and trypanosomes (Rottnest Island= 93, mainland= 28). Samples were screened for piroplasms, using a nPCR that amplifies a 850 bp fragment of the 18S ribosomal RNA gene (18S rDNA), as previously described (Jefferies, Ryan, & Irwin 2007) (see Appendix 10.4.2.1). Similarly, trypanosomes were detected using a nested PCR method, with trypanosome-specific primers targeting a variable region (~1,500 bp) of the trypanosome 18S rDNA, as previously described (McInnes, Hanger*, et al.* 2011) (see Appendix 10.4.2.2). Positive controls for piroplasm and trypanosome were kindly provided by Professor Una Ryan. PCR products were visualised in 1% agarose gels made of 0.5x TBE buffer, stained with SYBR-Safe (Life Technologies, Carlsbad, USA) and ran at 90 V, 400 mAmp for ~45 min. Bands of the expected molecular weight were excised with sterile scalpel blades using a dark light trans-illuminator (Clare Chemical Research, USA). DNA within the excised bands was purified using the filter tip method and sequenced using forward and reverse primers (see Appendix 10.4.3). All PCR and sequencing reactions were carried out in an Applied Biosystems (AB) GeneAmp 2720 Thermal Cycler. Sequencing reactions were subsequently purified using the ethanol precipitation method (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Scoresby, Australia) (see Appendix 10.4.3), and final sequencing was carried out on an ABI 3790 96 capillary automatic sequencer (Applied Biosystems, Scoresby, Australia) at SABC, Murdoch University. Primer sequences were removed and the remaining sequence information was analysed using 4Peaks version 1.7.2 (A. Griekspoor and Tom Groothuis, at http://nucleobytes.com/4peaks/.

7.3.2 ***Selection of reference individuals and samples***

Criteria for the inclusion of reference individuals was applied *a posteriori* and included: cloacal temperatures between 36.5 ˚C and 38.5 ˚C (Bartholomew 1956) taken immediately after anaesthesia induction, a dehydration status between 0-5% (see section 3.3), a body condition score between 1 and 3 (see section 3.3), mucous membrane appearance (pink or pale pink and moist), as well as the absence of other obvious signs of disease. Sample quality was recorded at the time of collection and analysis, and samples were excluded if

haemolysis and/or lipaemia were noted. Platelet aggregates observed on blood smears were used to determine which ADVIA-120® platelet values were to be removed from the dataset.

7.3.3 *Statistical analyses*

Haematology, BLC and PBCM (i.e. leukocyte and red blood cell) datasets, were explored visually with non-metric Multidimensional Scaling (nMDS) using a Bray-Curtis similarity measure (Bray & Curtis 1957) in PAST v. 3.02 (Hammer, Harper, & Ryan 2001). Variables in the first two datasets were fitted to an approximate normal distribution by transforming the data using BoxCox transform (STATISTICA v. 9.1, StatSoft Inc.). Analyses were performed on haematology and blood chemistry data sets separately due to differences in sample sizes between these datasets (some individuals were tested for either HMT or BLC, which would mean their exclusion from analyses of the entire dataset due to missing data). Data of the HMT and BLC datasets were range-standardised to a scale between 0 and 1; by contrast, data of PBCM were not range standardised given that it had a binary format (presence and absence). Two- or three-dimensional models were selected according to the model that had the lowest stress statistic.

Multidimensional scaling offers a visual interface of the similarity or dissimilarity of samples in the groups of interest, but does not include a test for statistical significance. For this reason, a two-way Analysis of Similarity (Two-way ANOSIM) (Clarke 1993) with 9,999 permutations for *R* statistics was run, to determine the significance of any differences detected in the HMT and BLC respond variables. The greater the number of permutations the greater the precision of the *p*-value obtained (Manly 2007). To establish the contribution of each dependant variable to the overall similarity or dissimilarity observed between groups, a pairwise similarity percentage (SIMPER) (Clarke 1993) analysis using the Bray-Curtis similarity measure (Bray & Curtis 1957) was carried out. SIMPER results (i.e. percent of contribution of each variable to the similarity or dissimilarity) are accompanied by the arithmetic mean ($\bar{x}$) and standard deviation (SD) for each HMT and BLC variable. For each PBCM observed in blood smears, a 95% confidence interval (CI) was calculated using the Wilson model for $n \leq 40$, and the Jeffreys model for $n \geq 40$ (Brown, Cai, & DasGupta 2001). A combined sample dataset (Rottnest Island and mainland), with *sex* and *site* as independent factors was carried out and then subsequent analyses were carried out for the Rottnest Island sample only, with *season* and *sex* as independent factors. The mainland sample was not subject to independent statistical analysis as the model was too uneven across sexes, season, and sample size at each

trapping location. Unless stated otherwise, PAST v. 3.02 statistical software package was used for all multivariate analyses, including multidimensional scaling modelling. Reference intervals for selected HMT and BLC analytes were explored and constructed using Reference Value Advisor v.2.1 (Geffre *et al.* 2011), a set of macroinstructions in Excel®. This tool, has been successfully used in constructing HMT and BLC references intervals for other species (Bourges-Abella *et al.* 2011 ; Geffré *et al.* 2011), (Bryant *et al.* 2012), and is accepted by the American Society for Veterinary Clinical Pathology as a tool that adheres to the most recent guidelines (C28-A3) (CLSI 2008) of the IFCC-CLSI. Although a test for normality (Anderson-Darling) was performed to all dependant analytes data, all datasets were transformed using the generalized Box-Cox transformation. Outliers and suspect data were detected with Dixon-Reed and Tukey's tests. The first detects a single outlier using the ratio of its distance to the closest value divided by the range of values (CLSI 2008). The second uses the median and the 25% and 75% percentiles of the interquartile range (CLSI 2008); 'suspect' data were always left in, unless there was an obvious clinical reason against the inclusion (e.g. mucopurulent nasal discharge, opisthotonus, ataxia).

As recommended by the revised CLSI guidelines, the non-parametric calculated reference interval is reported when the data of a sufficient sample size (i.e. ≥40, ideally ≥120) (CLSI 2008) has a unimodal distribution (Geffre *et al.* 2011). Confidence intervals for the non-parametric calculated reference interval were computed using a bootstrap method (Geffre *et al.* 2011). This approach was used to construct HMT and BLC reference intervals for the Rottnest Island subpopulation, as well as for females and males separately. Reference intervals for the mainland dataset (n< 40), were those given by the robust method with Box-Cox transformed data when possible; otherwise reference intervals are given using the robust method with untransformed data. In this case, confidence intervals were computed using a non-parametric bootstrap method (Geffre *et al.* 2011). In all cases, the corresponding lower and upper limit of the reference interval as well as the 2.5, 5, 90, and 97.5% confidence intervals are given where possible. Similarly, standard descriptive statistics are presented and include sample size, arithmetic mean ($\bar{x}$), median, standard deviation (SD), minimum and maximum. Whenever possible, these are given using the robust method of the untransformed data; otherwise the values provided are those of the standard method of the untransformed data.

Manual differential leukocyte counts data were used when calculating the corresponding reference intervals. To validate our choice, we calculated the correlation coefficients (*r*) using least squares linear regression analysis to compare the results of the ADVIA-120®

for NEUT, LYMPH, EOS, BASO and MONO, to those obtained through the manual differential count. Correlation between the two measurements was considered excellent if r≥ 0.95, very good if *r*= 0.85–0.94, good if *r*= 0.75–0.84, fair if *r*= 0.65–0.74, and poor if *r*≤ 0.64. For the Rottnest Island sample, the reference interval for platelet concentration was constructed after removing samples that contained platelet clumps on the blood smear. Comparison of HMT and BLC values between mainland locations was not done as the model was too uneven. An unpaired non-parametric Mann-Whitney U test (MWU) was used to explore significant differences between plasma concentrations of vitamin E from captive *S. brachyurus* (Perth Zoo) that were sourced from Rottnest Island, and plasma concentrations of vitamin E of free-ranging animals also from Rottnest Island. The data used for this analysis were obtained from both populations during the same season (i.e. winter). Separate MWU tests were used to determine significant differences in RBC between microfilariae-positive and microfilariae-negative animals on Rottnest Island and on the mainland, and significant differences in WBC and LYMPH between flower cell-positive and flower cell-negative animals on Rottnest Island. Chi-square analysis was used to determine variation in microfilariae and piroplasm prevalence across sites (Rottnest Island vs. mainland); as well as across sexes and seasons (only Rottnest Island). For this last case, an equal proportion of samples was assumed (i.e. expected values calculated assuming an equal distribution across the number of seasons). Chi-square was also used to determine significance in the variation of flower cells (only observed in Rottnest Island peripheral blood smears) across sex and seasons in the Rottnest Island sample. Odds ratio (OR) and its corresponding 95% CI when presented, were calculated using Woolf's method (1955). All other 95% CI for estimates of proportions (i.e. prevalence), were calculated using the Wilson model for n≤ 40, and the Jeffreys model for n≥ 40 (Brown, Cai, & DasGupta 2001). In all analyses, statistical significance was set to $p< 0.05$.

7.4 Results

There was a lack of agreement (*r*≤ 0.64) for EOS, BASO and MONO (Figure 7-2a-c) between the in house (200 cells) and the ADVIA-120® counts. By contrast, there was obvious agreement (*r*= 0.85-0.94) between estimates for NEUT and LYMPH (Figure 7-2d-e) of both the automated counter and the manual differential count. The ADVIA® 120 data included fewer EOS and more NEUT than the manual differential (Figure 7-2). Inspection of the manual differential data revealed that the ADVIA® 120 included EOS within the NEUT count. Discrepancies with the ADVIA-120® data are therefore likely to reflect the use of the 'multi-species' software using the default setting (canine), which would reduce sensitivity to features that distinguish between these cell populations.

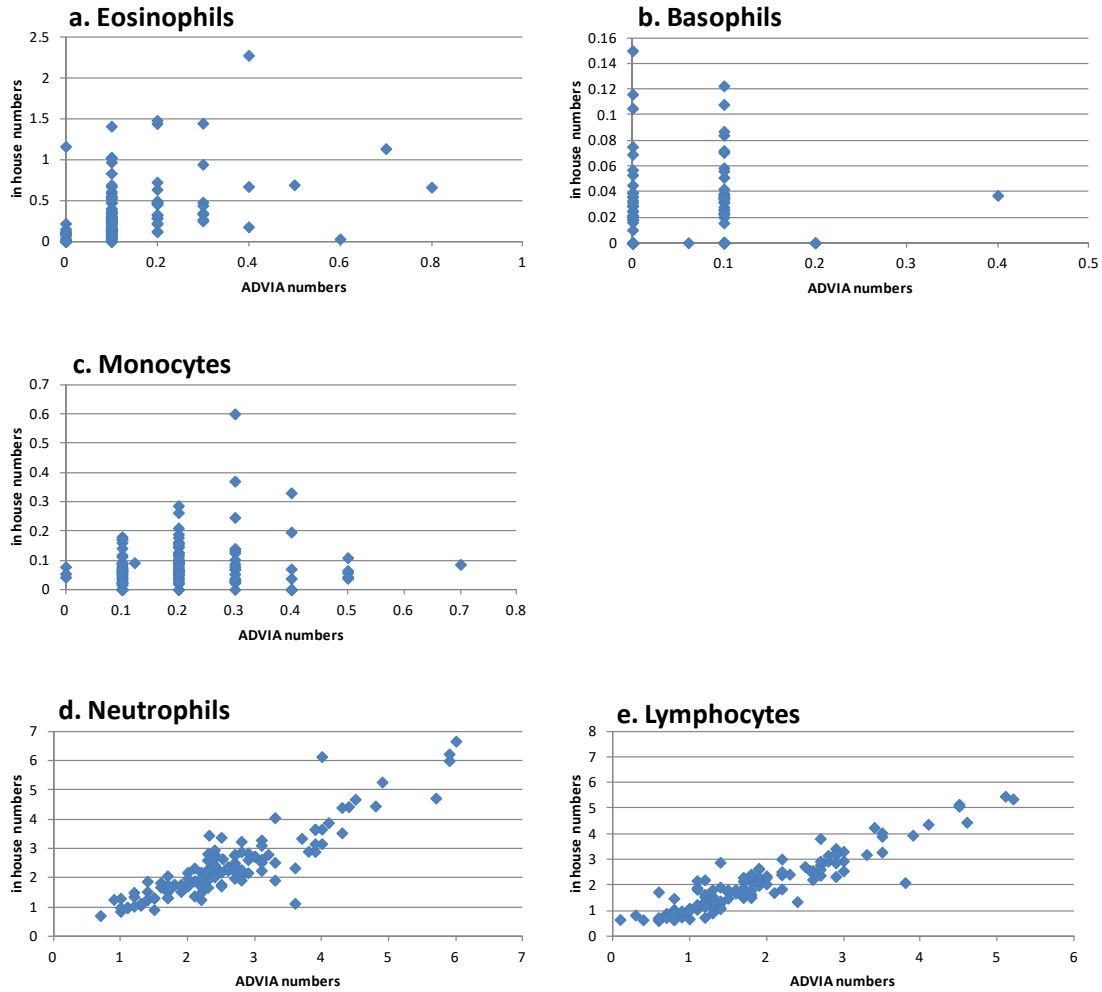


Figure 7-2 Correlation (*r*) of white blood cell classification by the ADVIA-120® with the 200-cell manual differential method. Eosinophils (a. *r*= 0.41), Basophils (b. *r*= 0.153), Monocytes (c. *r*= 0.04), Neutrophils (d. *r*= 0.90), and Lymphocytes (e. *r*= 0.93). Distances along the axes are unit-less, therefore the positions of the points in the plots are relative distances from one another rather than absolute differences read in these units.

7.4.1 *Correlates of HMT, BLC, and PBCM data, with site and sex for the combined data sample (Rottnest Island-mainland)*

There were significant difference in the HMT (ANOSIM, *p*= 0.003; Table 7-3a), BLC (ANOSIM, *p*= 0.001; Table 7-3b) and PBCM (ANOSIM, *p*= 0.001; Table 7-3c) data by *site* (i.e. Rottnest Island and mainland), with an evident clustering of both HMT and BLC datasets by site (Figure 7-3a-b). By contrast, there were no differences in the HMT, BLC or PBCM data by *sex* (ANOSIM; Table 7-3a-c). Clustering was not evident for the PBCM datasets (Figure 7-3c).

Differences between Rottnest Island and mainland animals were more evident (i.e. Ct% >10; in order of greatest effect to least effect) for RBC, LYMPH, and CK (SIMPER; Table 7-4a-b), while differences in PBCM were more evident for Heinz bodies, acanthocytes, Rouleaux formation, and hypochromasia (SIMPER; Table 7-5). However, generally mainland animals had a greater RBC, PCV, HGB, CHCM, as well as NEUT and BASO counts, than those on Rottnest Island, which had greater LYMPH, EOS and WBC, and also a greater MCV. The BLC profiles of animals on the mainland and animals on Rottnest Island showed considerable differences. Except ALP, all assessed analyte plasma concentrations were higher for mainland *S. brachyurus* compared to Rottnest Island animals. The differences in CK, vitamin E, AST, ALT, and UREA in plasma are worth noting. For instance, animals on the mainland had CK readings almost 10 times higher than those of animals on Rottnest Island. There were also marked differences in polychromasia, anisocytosis, and poikilocytosis.

Table 7-3 Two-way ANOSIM of selected HMT variables (a) (corrected WBC, RBC, HGB, PCV, and absolute counts for leukocytes obtained with a manual differential on a blood smear), BLC analytes (b) (ALP, ALT, AST, CK, PROT, ALB, GLOB, CALC, PHOSP, CHOL, BILT, GLUC, CREAT, UREA and Vitamin E), and PBCM (c) across site and sex, for *S. brachyurus*. Bray-Curtis similarity index, Permutation N= 9,999. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

	Variables	Factor	R	*p*
a.	HMT	Site	0.296	**0.003**
		Sex	0.045	0.267
b.	BLC	Site	0.463	**0.001**
		Sex	-0.052	0.681
c.	PBCM	Site	0.434	**0.001**
		Sex	-0.043	0.522

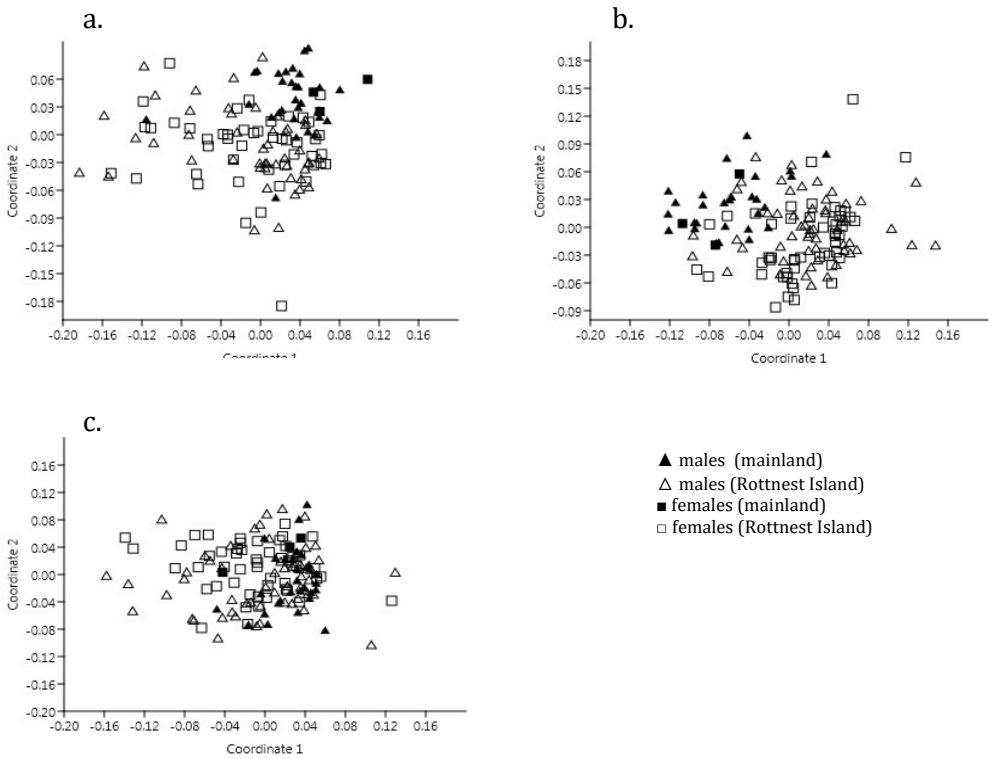


Figure 7-3 Non-metric MDS plots with Bray-Curtis similarity measure, illustrating the structural dissimilarity in HMT (a. stress statistic= 0.278), BLC (b. stress statistic= 0.312), and PBCM (c. stress statistic= 0.321) between Rottnest Island (open symbols) and mainland (filled symbols) *S. brachyurus* by site. Key legend applies for all plots. Note that the distances along the axes are unit-less, therefore the positions of the points in the plots are relative distances from one another rather than absolute differences read in these units. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

Table 7-4 SIMPER analysis indicating the contribution of specific variables to the observed *site* [Rottnest Island (RI) and mainland (ML)] differences in (a) HMT and (b) BLC profiles of *S. brachyurus*. HMT= haematology, BLC= blood chemistry, Ct= Percent of contribution to difference.

				RI		ML	
		Taxon	Ct %	$\bar{x}$	SD	$\bar{x}$	SD
a.	HMT	RBC ($x10^{12}$/L)	10.7	5.84	0.87	7.38	1.01
	(27.64) †	Lymphocytes ($x10^9$/L)	10.1	2.09	1.09	1.64	1.08
	RI n= 96	Eosinophils ($x10^9$/L)	9.86	0.40	0.365	0.14	0.214
	ML n= 32	Neutrophils ($x10^9$/L)	9.86	2.23	0.913	2.92	1.52
		PCV (%)	9.61	33.4	4.46	40.9	4.08
		Basophils ($x10^9$/L)	9.32	0.02	0.03	0.03	0.03
		HGB (g/L)	9.06	108	15.3	134	15.5
		Monocytes ($x10^9$/L)	8.79	0.08	0.08	0.08	0.07
		WBC ($x10^9$/L)	8.24	4.82	1.58	4.78	1.40
		MCV (fL)	7.43	61	3.65	59.8	3.27
		CHCM (g/L)	7.01	307	15.5	326	27.2
b.	BLC	CK (U/L)	11.2	942	1,331	7,665	6,799
	(22.76) †	ALT (U/L)	9.80	217	62.2	436	156
	RI n= 106	Vitamin E (mg/L)	9.10	6.53	1.80	9.93	2.73
	ML n= 32	ALP (U/L)	7.62	8,204	10,094	8,074	13,947
		AST (U/L)	7.03	49.8	31.6	239	308
		Phosphorus (mmol/L)	6.63	1.19	0.467	1.68	0.67
		Protein (g/L)	6.62	60.4	4.62	63.9	4.35
		Calcium (mmol/L)	6.35	2.20	0.201	2.47	0.141
		Glucose (mmol/L)	6.07	4.15	2.21	5.46	2.33
		Cholesterol (mmol/L)	6.00	2.80	0.539	2.99	0.65
		Albumin (g/L)	5.76	36.3	1.91	38.9	2.19
		Bilirubin (µmol/L)	5.26	4.29	1.65	5.32	2.75
		Globulin (g/L)	5.04	24.1	3.75	24.9	3.56
		Urea (mmol/L)	4.43	6.87	1.53	9.60	4.94
		Creatinine (µmol/L)	3.16	70.9	16	83.3	23.7

RBC= red blood cell concentration, HGB= haemoglobin, PCV= packed cell volume, CHCM= corpuscular haemoglobin concentration mean, MCV= mean corpuscular volume, WBC= white blood cell count, CK= creatine kinase, ALT= alanine aminotransferase, ALP= alkaline phosphatase, AST= aspartate aminotransferase, GGT= gamma-glutamyl transferase

† Overall average dissimilarity.

Table 7-5 SIMPER analyses indicating the contribution to the observed *site* [Rottnest Island (RI) and mainland (ML)] differences in specific PBCM in *S. brachyurus*. Overall average dissimilarity= 29.01. PBCM= peripheral blood cell morphology, Ct= percent of contribution to difference, CI= confidence interval.

Taxon	Ct %	RI (n=107)	95% CI *	ML (n= 34)	95% CI **
Heinz Bodies	11.4	75 (70.1%)	0.61-0.78	20 (59%)	0.42-0.74
Acanthocytes	11	52 (49.0%)	0.39-0.58	19 (56%)	0.39-0.71
Rouleaux Formation	10.9	46 (43%)	0.34-0.52	17 (50%)	0.34-0.66
Hypochromasia	10.3	96 (90%)	0.83-0.94	19 (56%)	0.39-0.71
nRBCs	8.04	97 (91%)	0.84-0.95	23 (68%)	0.51-0.81
Anisocytosis	8.03	86 (80%)	0.72-0.87	25 (73.5%)	0.57-0.85
Echinocytes	7.88	30 (28%)	0.20-0.37	7 (21%)	0.10-0.37
Polychromasia	7.51	98 (92%)	0.85-0.96	24 (71%)	0.54-0.83
Schistocytes	6.40	26 (24.3%)	0.17-0.33	4 (12%)	0.05-0.27
Poikilocytosis	5.45	93 (87%)	0.80-0.92	29 (85%)	0.70-0.94
Keratocytes	4.91	24 (22.4%)	0.15-031	1 (3%)	0.01-0.15
Flower Cells	4.52	23 (21.5%)	0.14-0.30	0 (0%)	0.00-0.10
Howell-Jolly Bodies	3.42	99 (92.5%)	0.86-0.96	31 (91%)	0.77-0.97
Reactive Lymphocytes	0.29	106 (99.1%)	0.96-0.99	34 (100%)	0.89-1.00

* calculated using Jeffreys model (Brown, Cai, & DasGupta 2001)

** calculated using Wilson's model (Brown, Cai, & DasGupta 2001)

7.4.2 *Correlates of HMT, BLC and PBCM data, with sex and season for Rottnest Island S. brachyurus*

There were significant differences in the HMT (ANOSIM, p= 0.001; Table 7-6a), BLC (ANOSIM, p= 0.001; Table 7-6b) and PBCM (ANOSIM, p= 0.001; Table 7-6c) data of Rottnest Island animals by *season*. However, clustering was not that evident in the non-parametric multidimensional scaling plot (Figure 7-4a-c). By contrast, there were no differences in HMT, BLC and PBCM data by sexes (ANOSIM; Table 7-6a-c). Seasonal differences in the HMT of Rottnest Island animals were more evident (i.e. Ct% >10; in order of greatest effect to least effect) for MONO, LYMPH, WBC, and MCV (SIMPER; Table 7-7a); while seasonal differences in their BLC were more evident (i.e. Ct% >8; in order of greatest effect to least effect) for vitamin E, CK, PROT, and GLUC (SIMPER; Table 7-7b). With the exception of MONO, and some mild variations across HMT analytes (e.g. NEUT and BASO counts and RBC) there appeared to be a general decrease trend for all other variables that would start in spring and carry on until winter when values will start to rise (Figure 7-5). Although slight variations were present in the BLC profiles of Rottnest Island animals, a general seasonal decreasing pattern that would start either in winter, spring or summer, was observed for all analytes measured (Figure 7-6). Similarly, significant seasonal variations in the PBCM populations of Rottnest Island animals were observed (Table 7-6), with differences being more evident (i.e. Ct% >11) for Heinz bodies, acanthocytes and Rouleaux formation (Table 7-8). Photomicrographs of some red blood cell morphologies are presented in (Figure 7-7).

Table 7-6 Two-way ANOSIM of selected HMT variables (a) (corrected WBC, RBC, HGB, PCV, and absolute counts for leukocytes obtained with a manual differential on a blood smear), BLC analytes (b) (ALP, ALT, AST, CK, PROT, ALB, GLOB, CALC, PHOSP, CHOL, BILT, GLUC, CREAT, UREA and vitamin E), and PBCM (c) across season and sex, for *S. brachyurus* on Rottnest Island. Bray-Curtis similarity index, Permutation N= 9,999. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

	Variables	Factor	R	*p*
a.	HMT	Season	0.183	**0.001**
		Sex	0.039	0.084
b.	BLC	Season	0.160	**0.001**
		Sex	0.023	0.190
c.	PBCM	Season	0.304	**0.001**
		Sex	0.035	0.114

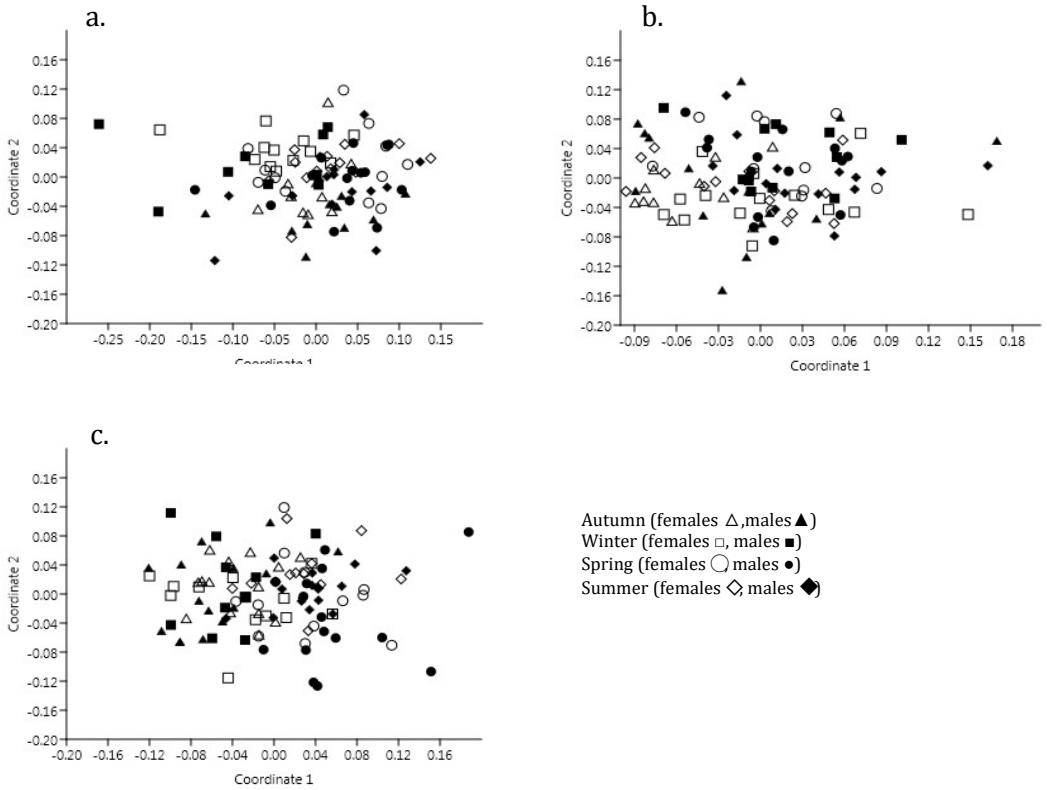


Figure 7-4 Non-metric MDS plots with Bray-Curtis similarity measure, illustrating the structural dissimilarity of HMT (a. stress statistic= 0.220), BLC (b. stress statistic= 0.262), and PBCM (c. stress statistic= 0.288) communities of animals on Rottnest Island between seasons (autumn: triangles, winter: squares, spring: circles, and summer: diamonds). Key legend applies to both plots. Note that the distances along the axes are unit-less, therefore the positions of the points in the plots are relative distances from one another rather than absolute differences read in these units. HMT= haematology, BLC=blood chemistry, PBCM= peripheral blood cell morphology.

Table 7-7 SIMPER analysis indicating the contribution of specific variables to the observed seasonal differences in (a) HMT and (b) BLC profiles of *S. brachyurus* on Rottnest Island (RI). HMT= haematology, BLC=blood chemistry, Ct: Percent of contribution to difference.

				Autumn		Winter		Spring		Summer	
		Taxon	Ct %	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
a.	HMT	Monocytes (x10^9/L)	11.6	0.112	0.112	0.091	0.064	0.068	0.068	0.052	0.045
	(24.62) †	Lymphocytes (x10^9/L)	11.5	2.032	0.840	1.56	0.750	2.38	1.29	2.24	1.16
	RI n= 96	WBC (x10^9/L)	10.9	4.55	1.25	4.052	1.201	5.53	1.75	4.88	1.62
		MCV (fL)	10.9	57.6	2.59	65.2	2.96	60.4	2.59	60.9	2.56
		Basophils (x10^9/L)	9.68	0.019	0.035	0.020	0.022	0.022	0.031	0.013	0.026
		RBC (x10^{12}/L)	8.84	5.58	0.61	5.71	0.97	5.75	0.74	6.21	0.98
		Eosinophils (x10^9/L)	8.32	0.374	0.321	0.260	0.188	0.620	0.509	0.311	0.218
		Neutrophils (x10^9/L)	8.14	2.019	0.530	2.113	0.606	2.44	1.16	2.27	1.036
		PCV (%)	7.61	33	3.8	31	3.8	35	4.5	34	4.7
		HGB (g/L)	6.60	104	15	103	17	107	12	116	15
		CHCM (g/L)	5.84	323	22	294	6.8	306	11	306	5.9
b.	BLC	Vitamin E (mg/L)	9.11	7.71	1.86	6.82	1.77	5.69	1.65	6	1.27
	(19.72) †	CK (U/L)	8.95	1,071	1,249	783	589	1,040	1,040	878	1,984
	RI n= 106	Protein (g/L)	8.22	58.6	4.15	58.5	3.59	62.2	5.25	61.9	4.15
		Glucose (mmol/L)	8.14	4.23	1.79	2.95	2.03	4.34	1.96	4.95	2.57
		Cholesterol (mmol/L)	7.78	2.7	0.6	3	0.5	2.9	0.5	2.7	0.5
		Phosphorus (mmol/L)	7.43	1	0.4	1.2	0.4	1.5	0.5	1.2	0.5
		Globulin (g/L)	7.12	23.3	2.98	22.5	3.14	25.6	4.35	24.9	3.71
		ALT (U/L)	6.91	185	37	234	74.7	230	56.3	220	65.3
		Calcium (mmol/L)	6.51	2.29	0.197	2.20	0.172	2.11	0.162	2.20	0.23
		Bilirubin (µmol/L)	6.05	5.3	1.8	5	1.6	3.9	0.6	3.2	1.4
		Albumin (g/L)	5.47	35.3	2.15	36	1.36	36.7	2.01	37	1.63
		ALP (U/L)	5.41	7,582	11,043	4,622	2,361	9,390	10,168	10,787	12,492
		AST (U/L)	4.92	57.3	30.7	55.6	48.3	43.3	15.7	43.9	22.9
		Creatinine (µmol/L)	4.20	81	20	74	18	62	9.1	68	8.2
		Urea (mmol/L)	3.78	6.47	1.88	7.01	1.53	7.10	1.54	6.90	1.16

RBC= red blood cell concentration, HGB= haemoglobin, PCV= packed cell volume, CHCM= corpuscular haemoglobin concentration mean, MCV= mean corpuscular volume, WBC= white blood cell count, CK= creatine kinase, ALT= alanine aminotransferase, ALP= alkaline phosphatase, AST= aspartate aminotransferase, GGT= gamma-glutamyl transferase

† Overall average dissimilarity.

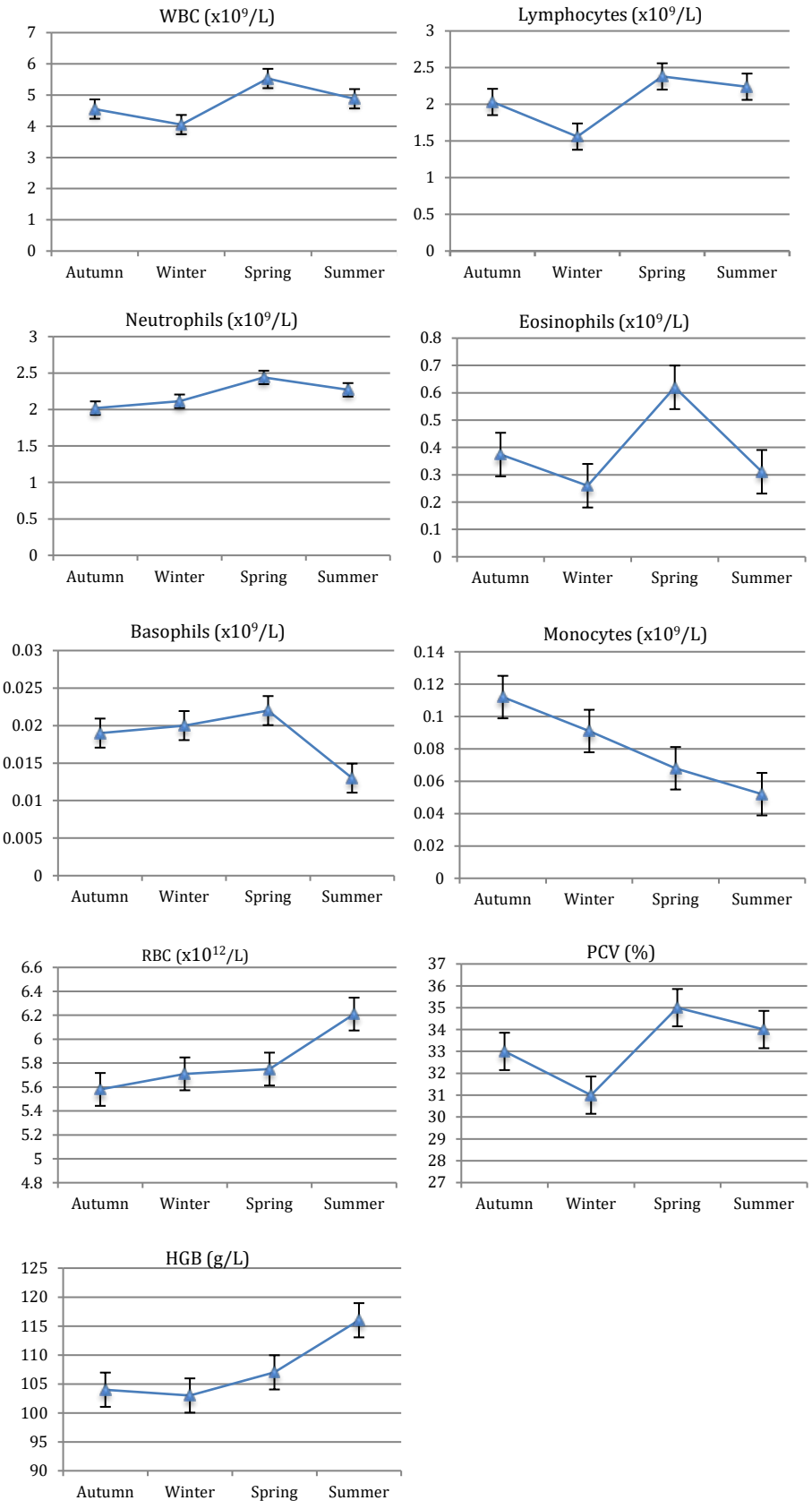


Figure 7-5 Marked linear plots for WBC, neutrophils, eosinophils, basophils, lymphocytes, monocytes, RBC, HGB and PCV across seasons for *S. brachyurus* trapped on Rottnest Island between March and December 2011. Autumn n= 20, Winter n= 21, Spring n= 27, Summer n= 28.

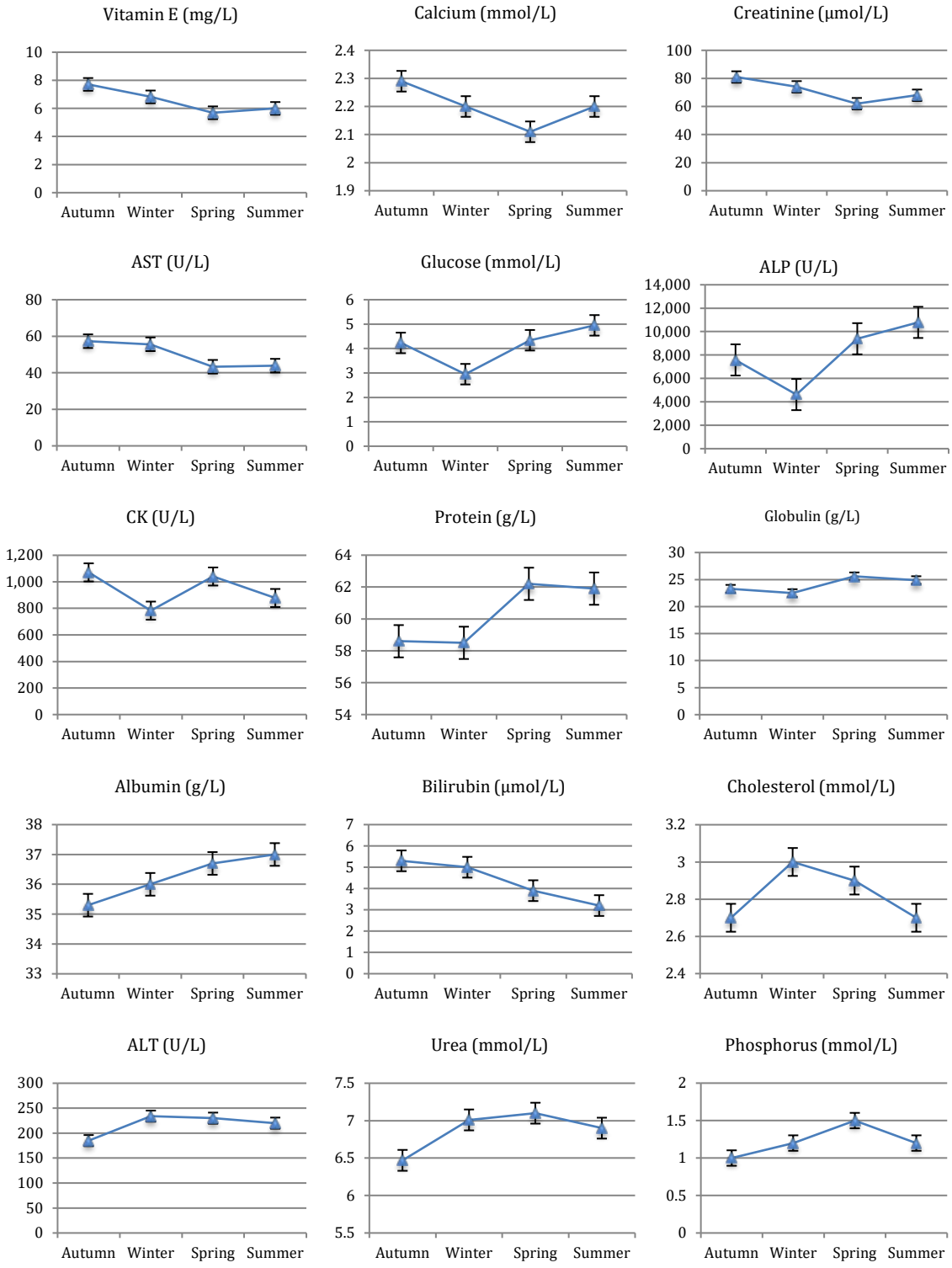


Figure 7-6 Marked linear plots for vitamin E, calcium, phosphorus, creatinine, urea, glucose, bilirubin, cholesterol, protein, albumin, globulin, AST, ALT, ALP and CK across seasons for *S. brachyurus* trapped on Rottnest Island between March and December 2011. Autumn n= 26, Winter n= 25, Spring n= 26, Summer n= 29.

Table 7-8 SIMPER analyses indicating the contribution to the observed seasonal differences in specific PBCM in *S. brachyurus* on Rottnest Island. Overall average dissimilarity= 25.5. PBCM= peripheral blood cell morphology. Ct= percent of contribution to difference, CI= confidence interval.

Taxon	Ct %	Autumn	95% CI *	Winter	95% CI *	Spring	95% CI *	Summer	95% CI *
Heinz Bodies	11.9	10 (36%)	21-54	17 (74%)	53-87	9 (33%)	19-52	22 (79%)	60-90
Acanthocytes	11.9	11 (39%)	24-58	15 (65%)	45-81	8 (30%)	16-48	18 (64%)	46-79
Rouleaux Formation	11.6	13 (46%)	29-64	15 (65%)	45-81	11 (41%)	24-59	7 (25%)	13-43
Echinocytes	9.16	11 (39%)	24-58	5 (22%)	9.0-42	6 (22%)	11-41	8 (29%)	15-47
Schistocytes	8.38	13 (46%)	29-64	3 (13%)	4.0-32	7 (26%)	13-45	3 (11%)	4.0-27
Flower Cells	8.26	5 (18%)	8.0-36	9 (39%)	22-59	12 (44%)	28-63	2 (7.1%)	2.0-23
Keratocytes	8.10	11 (39%)	24-58	9 (39%)	22-0.59	1 (3.7%)	1.0-18	3 (11%)	4.0-27
Anisocytosis	7.88	24 (86%)	68-94	23 (100%)	86-100	19 (70%)	51-84	20 (71%)	53-84
Poikilocytosis	5.77	26 (93%)	77-98	21 (91%)	73-98	19 (70%)	51-84	28 (100%)	88-100
Hypochromasia	4.61	24 (86%)	68-94	22 (96%)	79-99	22 (81%)	63-92	28 (100%)	88-100
nRBCs	4.43	28 (100%)	88-100	17 (74%)	53-87	24 (89%)	72-96	28 (100%)	88-100
Polychromasia	4.11	26 (93%)	77-98	21 (91%)	73-98	27 (100%)	87-100	24 (86%)	68-94
Howell-Jolly Bodies	3.37	27 (96%)	82-99	21 (91%)	73-98	25 (93%)	77-98	26 (93%)	77-98
Reactive Lymphocytes	0.58	28 (100%)	88-100	23 (100%)	86-100	26 (96%)	82-99	28 (100%)	88-100

* calculated using the Wilson model (Brown, Cai, & DasGupta 2001)
autumn n= 28, winter n= 23, spring n= 27, summer n= 28

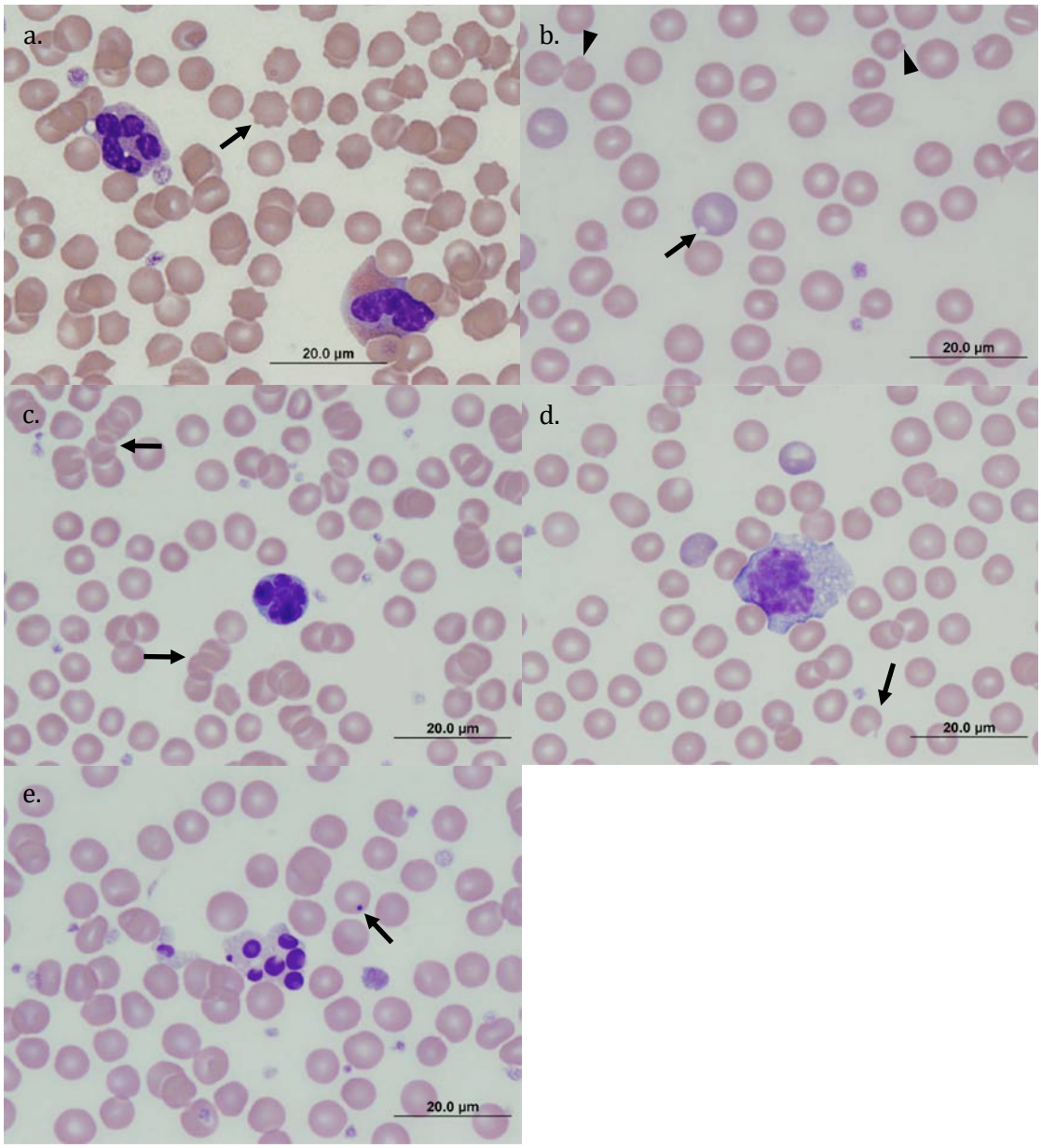


Figure 7-7 Some erythrocytic morphologies and intraerythrocytic inclusions found in peripheral blood smears of quokkas on Rottnest Island (also found in quokkas on mainland Western Australia). (a) echinocytes (arrow), two large cells present: neutrophil (left), eosinophil (right); (b) Heinz bodies (arrowheads), blister polychromatophilic cell (arrow), anisocytosis is also present in this plate; (c) Rouleaux formation (arrowheads), lymphocyte with a flower-like nucleus (large cell centre); (d) keratocyte (arrow), monocyte (centre) and polychromatophilic erythrocytes present; (e) Howell-Jolly body (arrow), leukocyte with fragmented nucleus (large cell centre). All images -original magnification x1000-, staining Wright-Giemsa.

7.4.3 *Reference intervals for HMT and BLC analytes for S. brachyurus on Rottnest Island and on the mainland*

Given the significant effect of *site* (see Table 7-3a,b,c) on the HMT and BLC profiles of *S. brachyurus,* reference intervals were calculated for Rottnest Island (Table 7-9) and the mainland (Table 7-10) separately. Although there was no significant effect of sex on the HMT and BLC parameters of animals on Rottnest Island (Table 7-6), HMT and BLC profiles of males and females were sufficiently different to warrant a separate set of reference intervals (see Appendix 10.4.4)

Table 7-9 Haematology and blood chemistry reference intervals for anaesthetised free-ranging quokkas (*S. brachyurus*) sampled on Rottnest Island (RI) between March and December 2011. Negative values of confidence intervals were interpreted as zero.

							Reference Intervals [a]		Confidence Intervals [b]			
Parameter (unit)	*n*	Mean [c]	Median [d]	SD [d]	Minimum [d]	Maximum [d]	Lower limit	Upper limit	2.5%	5%	90%	97.5%
RBC ($x10^{12}$/L)	113	5.83	5.81	0.86	4.14	8.51	4.26	7.69	4.14	4.56	7.46	8.51
HGB (g/L)	113	108	108	15.1	53	149	79	141	53	87	131	149
PCV (%)	101	33.6	34	4.5	20	47	23.1	43.9	20	27.1	40.9	47
CHCM (g/L)	112	307	306	14.8	281	342	284	339	281	287	336	342
MCV (fL)	113	61	60.5	3.71	52.7	71.2	53	70	52.7	55.4	67.1	71.2
Platelet ($x10^9$/L)	40	501	493	110	322	813	322	810	322	336	703	813
WBC (x109/L)	113	4.64	4.39	1.65	1.75	9.31	2.03	8.55	1.75	2.29	7.82	9.31
Neutrophils ($x10^9$/L)	106	2.09	2	0.73	0.82	4.65	0.93	4.01	0.82	1.05	3.38	4.65
Lymphocytes ($x10^9$/L)	108	1.99	1.76	1.09	0.59	5.36	0.62	5.12	0.59	0.67	4.16	5.36
Monocytes ($x10^9$/L)	106	0.07	0.06	0.06	0.00	0.26	**0.00**	0.25	**0.00**	**0.00**	0.18	0.26
Eosinophils ($x10^9$/L)	107	0.36	0.28	0.31	0.00	1.46	0.03	1.39	**0.00**	0.06	1.01	1.46
Basophils ($x10^9$/L)	105	0.01	0.00	0.02	0.00	0.09	**0.00**	0.07	**0.00**	**0.00**	0.06	0.09
CK (U/L)	108	754	433	700	138	3,240	153.7	3,045	138	182.8	2,251	3,240
ALT (U/L)	110	215	201	55.3	132	437	147	384	132	152	324	437
ALP (U/L)	103	5,757	5,620	3,064	1,387	17,880	1,517	13,272	1,387	1,792	10,208	17,880
AST (U/L)	107	45.1	40	17.7	13	108	25	97.2	13	26	84	108
GGT (U/L)	83	17.8	17	6.2	8	41	8.1	30	8	10	28.9	41
Protein (g/L)	111	60.2	59.8	4.62	49.5	72.3	50.6	70.3	49.5	53.3	68.8	72.3
Albumin (g/L)	111	36.2	36.3	1.92	30	41.1	31.9	39.8	30	32.8	39	41.1
Globulin (g/L)	111	24	23.5	3.78	14.4	36	15.7	32.4	14.4	18.6	30.7	36
Glucose (mmol/L)	111	4.15	3.70	2.18	0.6	12.5	0.68	9.94	0.60	1.06	7.94	12.5
Cholesterol (mmol/L)	111	2.83	2.80	0.54	1.5	4	1.88	3.82	1.50	2.1	3.70	4
Bilirubin (µmol/L)	110	4.28	4	1.72	1	9.4	1.82	9	1	2	7.79	9.4
Urea (mmol/L)	110	6.90	6.73	1.47	4.2	12.3	4.28	10.4	4.20	4.76	9.38	12.3
Creatinine (µmol/L)	110	71.3	68.7	15.6	41	112	47	110	41	49.6	104	112
Phosphorus (mmol/L)	111	1.20	1.20	0.46	0.4	2.8	0.48	2.34	0.4	0.6	1.94	2.80
Calcium (mmol/L)	111	2.19	2.18	0.19	1.63	2.69	1.8	2.64	1.63	1.91	2.51	2.69
Vitamin E (mg/L)	108	6.55	6.04	1.81	3.84	10.9	4.03	10.5	3.84	4.18	9.71	10.9

Erythrocyte variables obtained with ADVIA® 120. WBC values are those after correction with nucleated red blood cells. Polymorphonuclear cell values are those of the manual differential count. RBC= red blood cell concentration, HGB= haemoglobin, PCV= packed cell volume, CHCM= corpuscular haemoglobin concentration mean, MCV= mean corpuscular volume, WBC= white blood cell count, CK= creatinine kinase, ALT= alanine aminotransferase, ALP= alkaline phosphatase, AST= aspartate aminotransferase, GGT= gamma-glutamyl transferase

[a] reference intervals were computed using the non-parametric method (when n≥ 40).

[b] confidence intervals were computed using a bootstrap method (when 20 <n< 120).

[c] for the standard method of the untransformed data.

[d] for the robust method of the untransformed data.

Table 7-10 Haematology and blood chemistry reference intervals for anaesthetised free-ranging quokkas (*S. brachyurus*) sampled on the southwest of Western Australia (Jarrahdale, Collie and Walpole locations) between September 2010 and July 2011. Negative values of confidence intervals were interpreted as zero.

							Reference Intervals [a b c]		Confidence Intervals [d]			
Parameter (unit)	*n*	Mean [e]	Median [f]	SD [f]	Minimum [f]	Maximum [f]	Lower limit	Upper limit	2.5%	5%	90%	97.5%
RBC ($x10^{12}$/L) [c]	36	7.28	7.20	1.03	5.29	10.4	5.43	9.51	5.06	5.84	8.87	10.3
HGB (g/L) [a]	36	132	130	15.6	106	175	98.9	162	91.8	106	153	172
PCV (%) [b]	35	40.6	40	4.1	33	52	34	50.5	32.9	35.2	47.6	53.9
CHCM (g/L) [c]	36	326	318	28	290	391	261	375	250	277	356	391
MCV (fL) [c]	36	59.8	59.7	3.24	54	66.3	53.8	66.9	52.8	55.2	65.2	68.8
Platelet ($x10^9$/L) †	-	-	-	-	-	-	-	-	-	-	-	-
WBC ($x10^9$/L) [c]	36	4.79	4.64	1.43	2.45	7.8	2.43	8.16	2.12	2.85	7.20	9.16
Neutrophils ($x10^9$/L) [c]	33	2.95	2.91	1.54	0.65	6.19	0.41	6.68	0.15	0.87	5.73	7.66
Lymphocytes ($x10^9$/L) [b]	33	1.66	1.37	1.20	0.45	4.75	0.41	4.88	0.34	0.55	3.51	6.39
Monocytes ($x10^9$/L) [b]	32	0.07	0.07	0.05	0.00	0.16	**0.00**	0.18	**0.00**	0.01	0.15	0.21
Eosinophils ($x10^9$/L) [b]	32	0.11	0.08	0.11	0.00	0.37	**0.00**	0.38	**0.00**	**0.00**	0.28	0.50
Basophils ($x10^9$/L) [b]	33	0.03	0.02	0.03	0.00	0.11	**0.00**	0.14	**0.00**	**0.00**	0.08	0.25
CK (U/L) [c]	37	7,674	5,858	7,071	397	20,000	229	32,034	71,9	629	24,127	41,123
ALT (U/L) [c]	34	413	405	107	181	692	218	654	183	263	580	733
ALP (U/L) [c]	29	2,996	985	4,123	251	13,580	185	27,954	132	269	10,825	72,737
AST (U/L) [c]	34	175	139	119	57	448	48,6	544	42,6	58,3	392	709
GGT (U/L) [b]	24	19.9	16.1	10.6	8	44	6.7	48	5.8	8.5	36.6	60.1
Protein (g/L) [b]	37	63.8	63.8	4.63	53.7	71.9	53.8	72.6	51.1	56.4	70.7	74.5
Albumin (g/L) [c]	37	39.1	39	2.38	34.6	44.2	34.7	44.4	33.9	35.6	43	45.9
Globulin (g/L) [c]	37	24.7	24.6	3.62	17.6	33.1	18.1	32.8	16.9	19.5	30.7	34.9
Glucose (mmol/L) [c]	37	5.45	5.10	2.34	2	12.6	1.95	10.9	1.59	2.49	9.44	12.9
Cholesterol (mmol/L) [c]	37	2.99	2.92	0.72	1.8	4.4	1.70	4.60	1.50	1.93	4.21	4.99
Bilirubin (µmol/L) [c]	35	5.04	5.10	1.91	0.9	8	0.93	8.81	0.83	2.08	8.06	9.55
Urea (mmol/L) [c]	36	9.16	8.39	3.90	3.8	20.2	3.78	19.2	3.20	4.51	15.8	22.8
Creatinine (µmol/L) [c]	36	82.3	79.2	16.5	60	142	60.8	131	57.9	64.6	113	158
Phosphorus (mmol/L) [c]	37	1.68	1.56	0.72	0.7	3.5	0.62	3.42	0.52	0.79	2.91	3.97
Calcium (mmol/L) [c]	37	2.48	2.46	0.155	2.17	2.9	2.21	2.83	2.16	2.26	2.73	2.95
Vitamin E (mg/L) [c]	32	9.92	9.77	2.85	5.65	15.1	5.23	16.5	4.67	6.01	14.6	18.3

Erythrocyte variables obtained with ADVIA® 120. WBC values obtained after correction with nucleated red blood cells. Polymorphonuclear cell values obtained with manual differential count. RBC= red blood cell concentration, HGB= haemoglobin, PCV= packed cell volume, CHCM= corpuscular haemoglobin concentration mean, MCV= mean corpuscular volume, WBC= white blood cell count, CK= creatine kinase, ALT= alanine aminotransferase, ALP= alkaline phosphatase, AST= aspartate aminotransferase, GGT= gamma-glutamyl transferase

[a] RI computed using the robust method of the untransformed data, [b] RI computed using the standard method of the Box-Cox transformed data, [c] Reference intervals computed using the robust method of the Box-Cox transformed data, [d] Confidence intervals were computed using a bootstrap method (when 20 <n< 120), [e] for the standard method of the untransformed data, [f] for the robust method of the untransformed data, † Insufficient sample size: 90% or more of blood smears were positive to platelet clumps.

7.4.4 *Vitamin E (captive vs. free-ranging)*

A significant difference in plasma vitamin E between free-ranging *S. brachyurus* on Rottnest Island, and those in captivity at Perth Zoo ($U_{(35)}$= 34.5, Z= 3.01, p= 0.01) was found. Captive individuals had lower levels (Mdn= 4.15 mg/L) of circulating vitamin E than free-ranging animals (Mdn= 5.75 mg/L) (Figure 7-8).

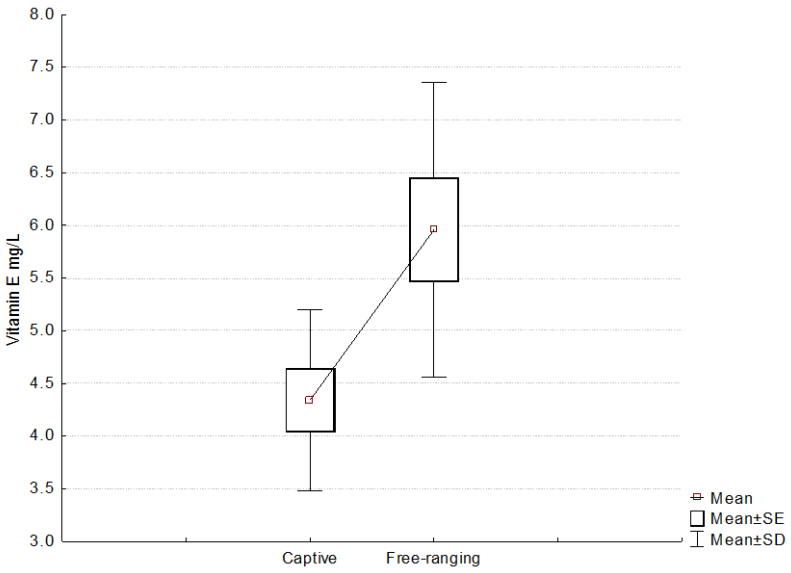


Figure 7-8 Box-plot of the mean concentration of vitamin E (mg/L) in plasma for *S. brachyurus* on Rottnest Island (free-ranging, n= 29) and *S. brachyurus* at Perth Zoo (captive, n= 8). Vitamin E was measured during winter.

7.4.5 *Haemoparasites*

The larvae of filarioid nematodes (microfilariae) were observed in both Rottnest Island and mainland quokkas. Microfilariae observed in blood smears from animals on Rottnest Island had a body length ranging from 202-273 µm (Figure 7-9a), while microfilariae in blood smears from animals on the mainland had a body length range of 135-142 µm (Figure 7-9b). All specimens, however, were anatomically similar, presenting a characteristic unsheathed, anucleated, long tapering tail, and four particularly obvious regions (under Wright-Giemsa staining): cephalic space, nerve ring, excretory vesicle and excretory cell, and anal vesicle (Figure 7-9), in agreement with microfilariae of the genus *Breinlia*. Parasitic intraerythrocytic inclusions (i.e. piroplasms) were also observed in animals from both subpopulations. Morphologically, we observed two types of piroplasms. The first type were ovoid (~1.7x0.9 µm or round (~1.7x1.7 µm, single, mostly located eccentrically within the cell, with obvious nuclear material and colourless cytoplasm, all enclosed by a thin membrane (Figure 7-9c,d). The second type were mostly oval or

pyriform in shape, ~0.7x0.4 µm, single or multiple, with obvious nuclear material but no apparent cytoplasm or membrane, and located eccentrically as well as centrically in the erythrocyte (Figure 7-9c). There were no piroplasmic inclusions observed in leukocytes.

Forty three of 146 *S. brachyurus* from Rottnest Island and mainland sites combined, were positive by light microscopy for the presence of microfilariae (prevalence of 29.4%, 95% CI 22.5-37.2), while 43 of 121 animals were positive by nPCR for the presence of piroplasms (prevalence= 35.5%, CI 27.4-44.3). There was no significant difference (χ^2_1= 1.00, p= 0.317) in the prevalence of microfilariae between Rottnest Island (32.1%, 35/109, CI 23.9-41.2) and mainland animals (21.6%, 8/37, CI 11.3-37.2) (Figure 7-10a). Similarly, site did not influence the probability of being positive to microfilariae (OR= 1.00, CI 0.19-1.08). *Theileria* sp. and *Babesia* sp. were detected in Rottnest Island animals by nPCR, with prevalences of 19.4% (18/93, CI 12.3-28.3) and 1.1% (1/93, CI 0.1-4.9) respectively. By contrast, only *Theileria* sp. was detected in mainland animals for a prevalence of 87.5% (CI 68.5-94.3). Overall, the prevalence of piroplasms was significantly (χ^2_1= 37.2, p= 0.001) higher in mainland animals (87.5%, 24/28) than in Rottnest Island animals (20.4%, 19/93) (Figure 7-10a), with the first (i.e. mainland) having a greater probability of being infected with piroplasms (OR 23.4, 95% CI 7.24-75.5). There were no sex differences in the prevalence of microfilariae (χ^2_1= 2.26, p= 0.131) (Figure 7-10b) or piroplasms (*Theileria* sp.: χ^2_1= 0.09, p= 0.925, *Babesia* sp.: χ^2_1= 0.01, p= 0.939) in Rottnest Island animals (Figure 7-10b). A significant difference (χ^2_3= 14.4, p= 0.002) in the prevalence of microfilariae across seasons was observed (Figure 7-10c), but not in the prevalence of *Theileria* sp. (χ^2_3= 0.46, p= 0.927) (Figure 7-10c). There were insufficient number of observations for *Babesia* sp. Significant differences in the PCV between microfilariae-positive and microfilariae-negative animals for Rottnest Island ($U_{(107)}$= 939, Z= 0.64, p= 0.709) and mainland samples ($U_{(33)}$= 83.5, Z= 0.346, p= 0.413), were not evident. Similarly, there were no significant differences in the RBC between microfilariae-positive and microfilariae-negative animals for Rottnest Island ($U_{(108)}$= 1197, Z= 0.72 p= 0.527) and mainland ($U_{(35)}$= 101, Z= 0.423, p= 0.779) groups. Both microfilariae and piroplasms were present in animals from all trapping locations on Rottnest Island as well as on the mainland (refer to Chapter 3, section 3.1). *Trypanosoma* spp. were not detected by light microscopy but were detected by nPCR (3/28, prevalence 10.7%, CI 3.7-27) in mainland samples, while all animals on Rottnest Island were negative.

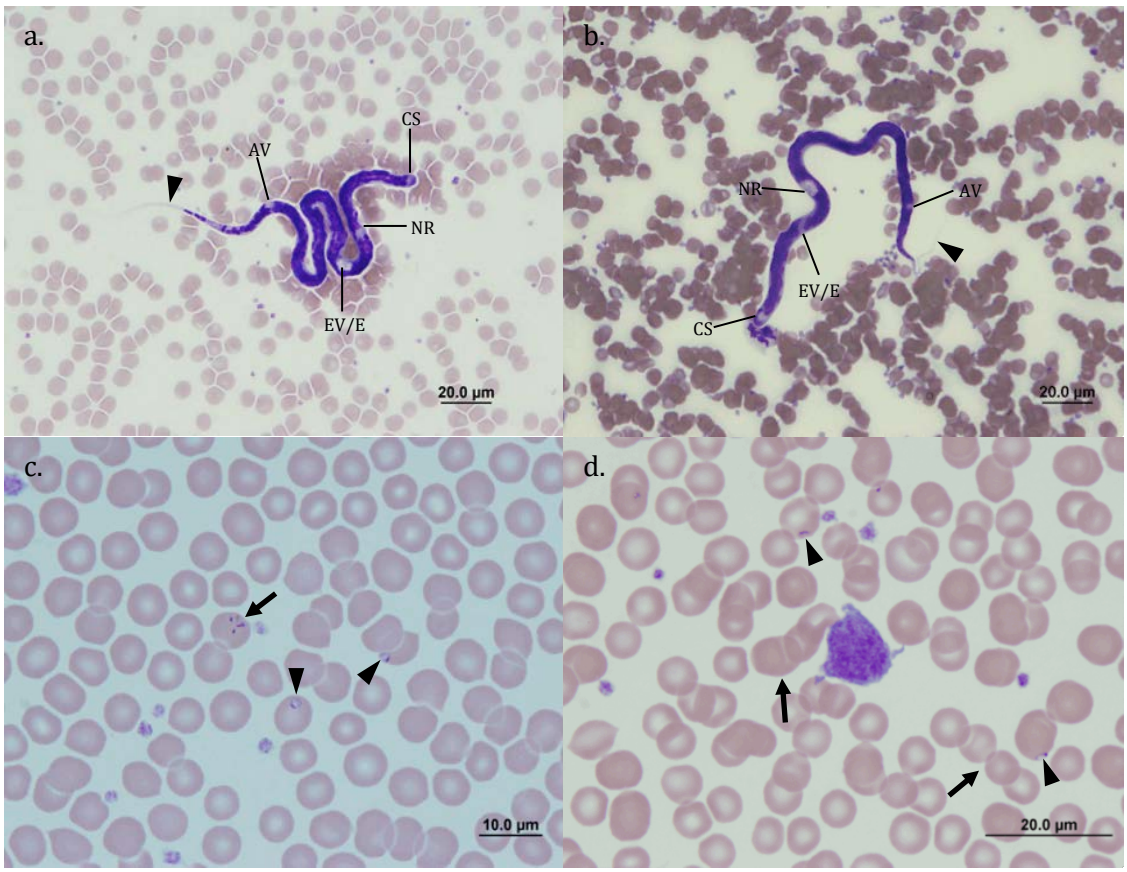


Figure 7-9 Photomicrographs of representative haemoparasites observed in peripheral blood smears of *S. brachyurus* on Rottnest Island and the mainland of Western Australia. (a) microfilaroid in blood smears of Rottnest Island animals, and (b) microfilaroid in blood smears of mainland animals; both images: original magnification x400, note the unsheathed, anucleated, long tapering tail (arrowheads) and AV= anal vesicle region, CS= cephalic space, EV/EC= excretory vesicle and excretory cell region, NR= nerve ring region; (c) two types of piroplasmic inclusions in red blood cells (arrowheads and arrow) and (d) piroplasmic inclusions in red blood cells (arrowheads), note the presence of a macroplatelet in the centre, Rouleaux formation (arrows), both images: original magnification x1000. All images stained with Wright-Giemsa.

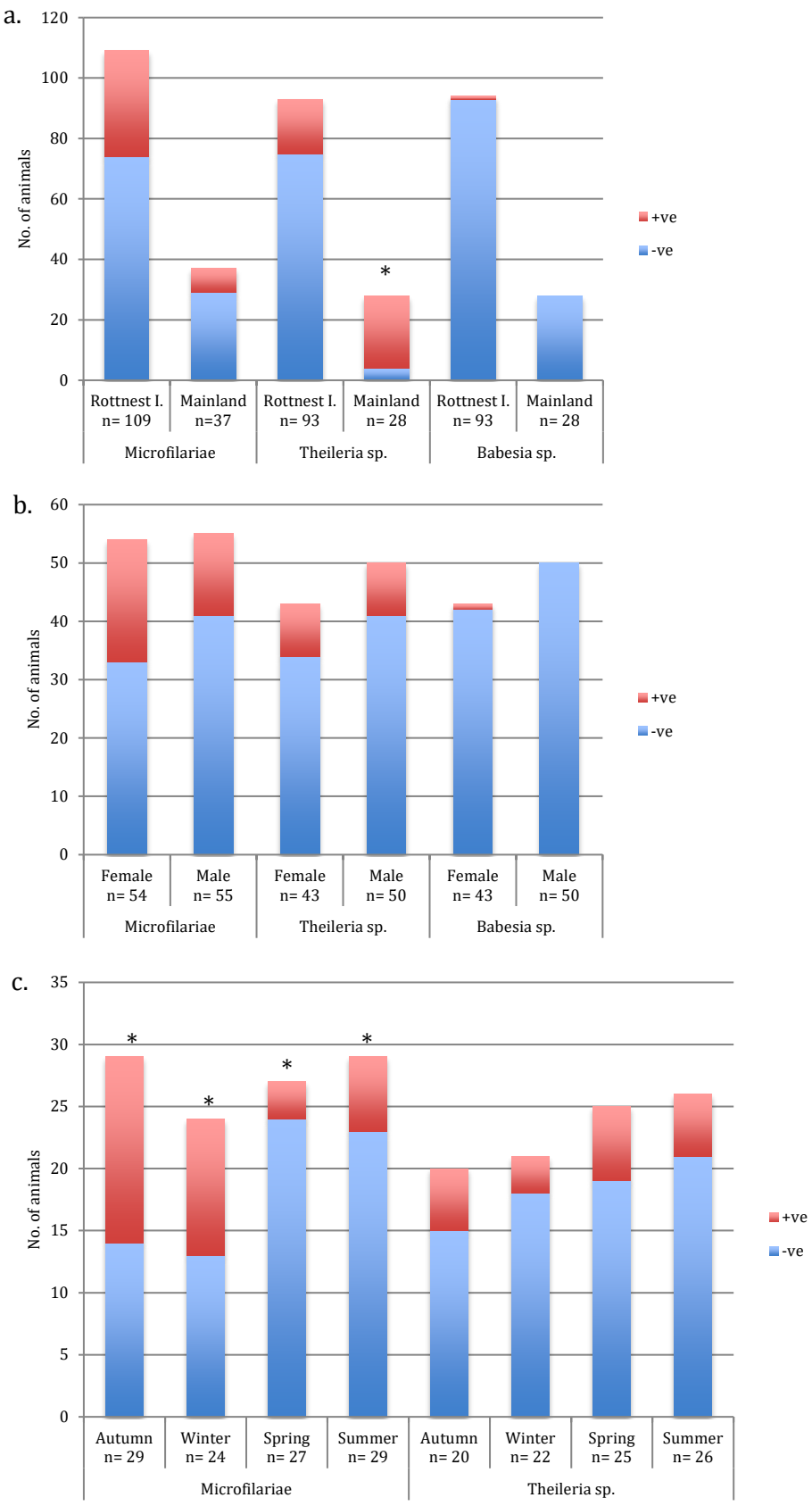


Figure 7-10 Prevalence plots. (a) prevalence of microfilariae and piroplasms by geographical location (Rottnest Island and mainland); (b) prevalence of microfilariae and piroplasms by sex (Rottnest Island sample); (c) seasonal distribution of animals positive and negative to microfilariae and *Theileria* sp. on Rottnest Island. (*) indicates significance at $p < 0.05$.

7.4.6 *Atypical cells*

Leukocytes exhibiting polylobated nuclei with condensed and homogeneous chromatin, resembling the petals of a flower (referred to as flower cells) (Figure 7-11a,b,c), as well as leukocytes with polylobated nuclei but lobes arranged in no specific shape (Figure 7-11d) were observed in peripheral blood of Rottnest Island animals. The prevalence of flower cells in Rottnest Island animals was estimated to be 21% (23/108, CI 14.4-29.7), with 1-6 cells per 200 leukocytes, and no significant sex (χ^2_1= 0.02, p= 0.892) or season (χ^2_1= 0.86, p= 0.354) differences in their prevalence. There were significant differences in the WBC between flower cell-positive (Mdn= 5.05 x 10^9/L) and flower cell-negative (Mdn= 4.27 x 10^9/L) animals ($U_{(108)}$= 668, Z= 0.49 p= 0.026), but not in the LYMPH count ($U_{(108)}$= 806, Z= 0.57 p= 0.201). By contrast, neither flower cells nor Atly were observed in peripheral blood of mainland animals.

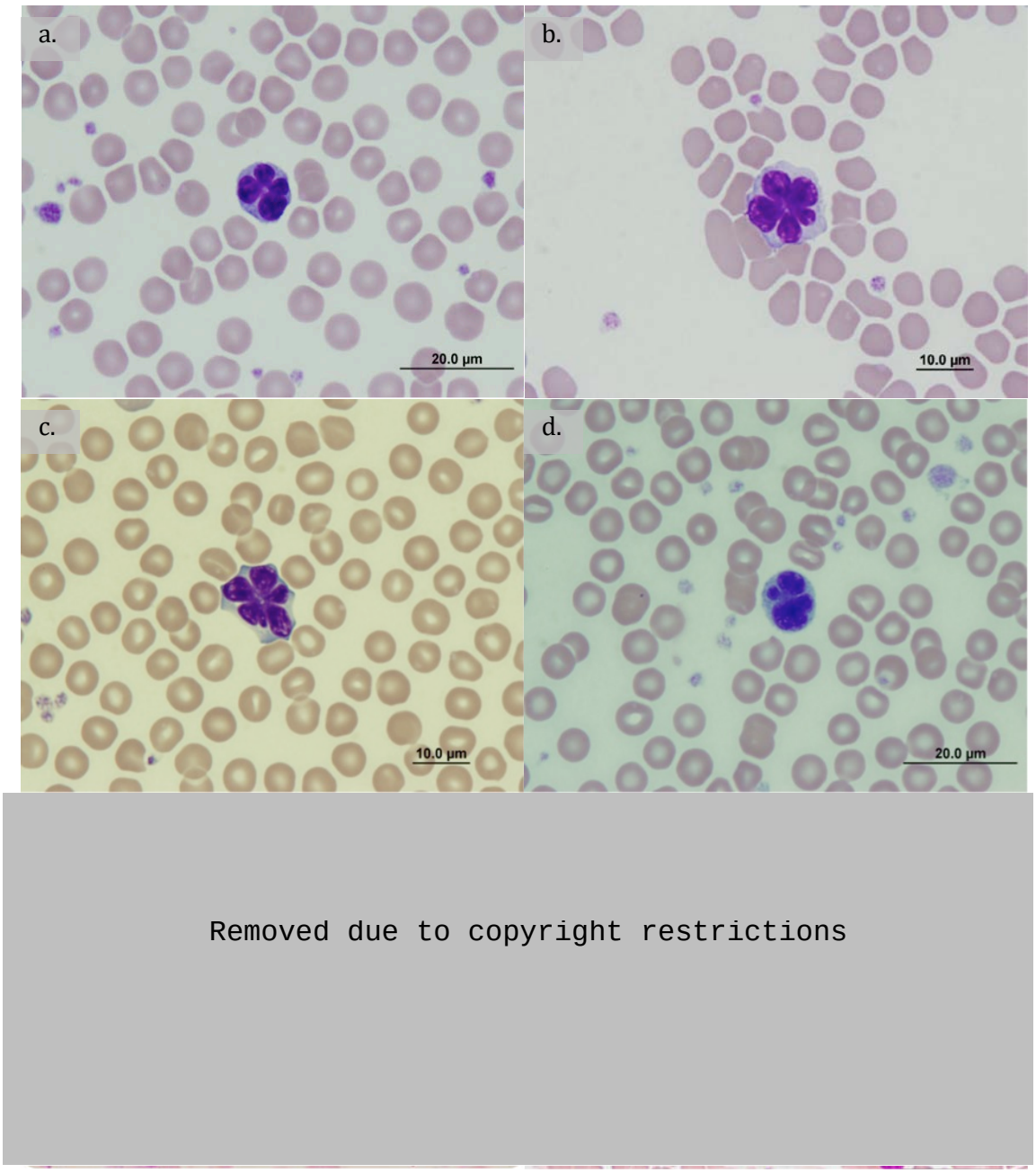


Figure 7-11 Microphotographs of atypical lymphocytes observed in peripheral blood smears of quokkas on Rottnest Island (a-d), of human patients with human T-cell lymphotrophic virus type 1 (HTLV-1) (e), and in mice infected with HTLV-1 (f). Quokka: (a-c) atypical lymphocytes exhibiting a "flower" shape-like polylobated nucleus with condensed homogeneous chromatin resembling flower cells in patients positive to HTLV-1; (d) atypical lymphocyte exhibiting a polylobated nucleus resembling "prototype lymphocytes" in human patients positive to HTLV-1; plates a-d: original magnification x1000, stain: Wright-Giemsa. Human: (e) pleomorphic "flower cells" (arrowheads) and "prototype cells" (arrow) observed in peripheral blood smears of a patient diagnosed with adult T-cell leukaemia [reproduced from Jain and Prabhash (2010)]. Mice: (f) pleomorphic "flower cells" (arrowheads) in peripheral blood of mice infected with HTLV-1 [reproduced from Tezuca et al. (2014)].

7.5 Discussion

Rottnest Island and mainland animals presented as significantly different subpopulations in their HMT and BLC. The Rottnest Island cohort exhibited an anaemic profile that had a seasonal pattern, with erythrogram parameters drastically decreasing during summer and gradually increasing during winter, but remaining significantly lower than those of animals on the mainland. This was also accompanied by PBCMs indicating a regenerative response from the bone marrow, and oxidative damage of erythrocytes. Overall, the mainland cohort showed an erythrogram with higher values than those of the Rottnest Island cohort. Though oxidative damage was also seen in erythrocytes of mainland animals, the prevalence of these abnormal cell morphologies was significantly lower than in the Rottnest Island group. Microfilariae of the genus *Breinlia* sp. were observed in blood smears of both cohorts, however, the prevalence was significantly greater in the Rottnest Island group. Both cohorts (Rottnest Island and mainland) were positive to piroplasms, however, *Theileria* sp. and *Babesia* sp. were detected in the Rottnest Island group where as *Theileria* sp. was the only one detected in the mainland group. Atypical lymphocyte morphologies associated with proliferative disorders of the haematopoietic and lymphoid tissues in other species, were detected in Rottnest Island animals but not on animals from the mainland. Mainland animals presented significantly greater plasma concentrations of CK, ALT, AST, ALP than their conspecifics on Rottnest Island. Analysis of vitamin E in plasma revealed significantly greater concentrations in free-ranging individuals than those in captivity (Perth Zoo). Overall, site and season had a significant input on the parameters measured, while sex had no influence.

In our study, the HMT of *S. brachyurus* on Rottnest Island and on the mainland, was found to be significantly different. This is in contrast with the findings of Clark and Spencer (2006), that found no significant difference in the HMT profiles of free-ranging *S. brachyurus* on Bald Island (east of Albany) and that of animals trapped at two unspecified locations in the Albany region (~418 km SE of Perth). Although not irrefutable, the absence of significance in the study by Clark and Spencer (2006), even in the presence of similar handling protocols (i.e. Bald Island and Albany groups), may be the result of low precision and sensitivity of the model due to a small sample size (Bald Island, n= 7; and mainland, n= 5) (Wobeser 2007, p. 124). Significant differences in HMT parameters between subpopulations of free-ranging Australian marsupials have been previously reported. Examples of these include the study by Clarke et al. (2013) on western ringtail possums (*Pseudocheirus occidentalis*) at Busselton, Tuart Forest National Park and

Gelorup all approximately 200 km SW of Perth, WA; the study by Pacioni et al. (2013) on woylies (*Bettongia penicillata ogilbyi*) trapped at Perup and Kingston subpopulations just NE of the town of Manjimup (~307 km south of Perth), as well as studies on euros (*M. robustus*) (King & Bradshaw 2010) and separate populations of captive parma wallabies (*M. parma*) in Australia (Clark *et al.* 2003).

The means for RBC, PCV, CHCM, HGB, WBC, NEUT and BASO counts, as well as all plasma chemistry analytes except GLOB, were consistently higher in mainland animals than on Rottnest Island. By contrast, Rottnest Island animals had higher LYMPH and EOS counts, and a greater MCV and GLOB in plasma as well. Differences between the HMT and BLC profiles of Rottnest Island and mainland animals could be attributed to the interplay of multiple factors such as site, climate, nutrition, social structure, age, underlying disease (e.g. inflammation, neoplasia), or the presence and absence of predators as it has been suggested for other species (Bennett *et al.* 2007 ; Clark 2004 ; Clark *et al.* 2003 ; Clarke *et al.* 2013 ; King & Bradshaw 2010 ; McKenzie, Deane, & Burnett 2002 ; Pacioni *et al.* 2013 ; Robert & Schwanz 2013). Alternatively, this difference may have also been influenced by stress due to the use of slightly different protocols in each subpopulation: Rottnest Island (all procedures under anaesthesia), mainland (anaesthetised after prolonged in-trap times and physical restraint for semi-invasive procedures such as ear tagging). Mainland animals were sourced from an ongoing population study and consequently it was not possible to standardise the capture methods between the two populations.

Higher HMT and BLC measures have been previously reported in animals where procedures were carried out under physical restraint only, including macropods (Barnes, Goldizen, & Coleman 2008 ; Crooks *et al.* 2003 ; Vogelnest & Portas 2010). In the absence of obvious signs of disease, alterations in HMT and BLC data may be attributed to the effects of greater levels of catecholamines (adrenaline and noradrenaline) and endogenous glucocorticoids (e.g. cortisol) that resulted from prolonged stress and physical restraint (Harvey 2012). However, it is also possible that the effect of the adrenal-mediated response may have been reversed when the quokkas were eventually anaesthetised (Vogelnest & Portas 2010). Consequently, the alteration in their erythrograms, leukograms and BLC may have been reversed, either partially or completely. Nevertheless, given the absence of data to compare the degree of the adrenomedullary (e.g. adrenaline or noradrenaline) and adrenocortical responses (e.g. cortisol) between Rottnest Island and mainland animals, it is unclear whether or not these endocrine factors influenced the results. Despite this, erythrograms and leukograms of animals on Rottnest Island and the mainland, as well as most of the BLC analytes, fell within the typically

reported values for other apparently healthy macropod species (Vogelnest & Portas 2010), including *S. brachyurus* (Clark 2004). However, all of the HMT parameters for both Rottnest Island and mainland animals were consistently lower than that of apparently healthy captive quokkas at Taronga Zoo (Vogelnest & Portas 2010).

Free-ranging *S. brachyurus* on Rottnest Island were found to have a seasonal erythrogram and erythrocyte morphologies indicative of anaemia. Seasonality was observed in RBC, PCV, HGB and MCV values. These values were found to decrease drastically during summer and gradually increase during winter on Rottnest Island. With the exception of MCV, all erythrogram parameters were significantly lower than those of the mainland group. This is in agreement with previous studies that determined a seasonal anaemia in the Rottnest Island quokka (Barker *et al.* 1974 ; Shield 1971), condition that was attributed primarily to nutritional factors such as low dietary protein. Anaemia has been documented in macropods under nutritional deficiencies (Barker *et al.* 1974), nematode infestation (Arundel, Barker, & Beveridge 1977) and unknown causes (Billiards, King, & Agar 1999). It is also in agreement with previous studies that have reported significant differences in the erythrocytic parameters between island macropods and their mainland conspecifics. For example, King and Bradshaw (2010) determined significant differences in RBC, PCV, HGB, MCV, and MCHC (CHCM in this study) values between island and mainland euros (*Macropus robustus* spp.), with island animals having lower values. They considered this profile as been characteristic of a normocytic hypochromic anaemia. In this study, similar MCV values between Rottnest Island (61 fL) and mainland (59.8 fL) subpopulations suggest a normocytic anaemia, while a lower CHCM suggests that this anaemia is also hypochromic. The higher prevalence of hypochromic and polychromatophilic erythrocytes, coupled with the higher proportion of nRBCs (per 1,000 erythrocytes) in peripheral blood smears of the Rottnest Island group compared to the mainland (SIMPER; Table 7-5), further supported this, and is consistent with a regenerative profile.

The higher prevalence (SIMPER; Table 7-5) and degree of severity of erythrocyte morphologies indicative of oxidative damage (i.e. Heinz bodies and keratocytes: Figure 7-7b,d,) in the Rottnest Island cohort than in the mainland cohort, coincided with the lowest erythrogram parameters of the first (HGB, PCV and RBC: Figure 7-5; Heinz bodies: Table 7-8), indicating a possible contribution of these oxidative processes to the overall anaemic state. According to Thrall (2012c), Heinz bodies (oxidised or denaturised HGB) may contribute to anaemic conditions by making the erythrocyte more susceptible to extravascular and intravascular haemolysis. The reason for the presence of Heinz bodies

in the quokka is unknown. However, the lower concentration of vitamin E (antioxidant) in plasma in the Rottnest Island group compared to the mainland group could in part explain the oxidative injury to HGB. Additionally, consumption of plants from the *Brassica* genus, family Brassicaceae, as well as other plants (e.g. garlic, onion: Amaryllidaceae; red maple: Sapindaceae) have been known to be responsible for oxidative injury to the erythrocyte (Thrall 2012c). Interestingly, members of all these three families of vascular plants appeared to be present on Rottnest Island or have been present at some point in time (Rippey, Hislop, & Dodd 2003), yet, according to a recent study on the diet components of quokkas on the island by Poole et al. (2014), plants belonging to these three families (Amaryllidaceae, Brassicaceae, Sapindaceae) were not found in faecal pellets of quokkas in Rottnest Island between April and May of 2011. Whether quokkas on the island consume these plants during other times of the year, is unknown.

Infection in *S. brachyurus* with microfilariae was first observed by Plimmer (1914) in a captive individual at the London Zoo. The infection was then reported in animals from Rottnest Island (Wahid 1962 ; Yen 1983), and according to Wahid (1962), the species involved was *Breinlia macropi*. The infection was considered to be hyperendemic, closed (involving only one microfilariae species) and with a prevalence between 33-61% (Yen 1983). Our results are in agreement with this, as the morphology of all microfilariae observed in blood smears was consistent with that of *Breinlia* sp. given the obvious absence of a sheath, and a long taping, anucleated tail (Clark 2004). However, adults were not available to establish the final identity of this microfilaria in the current study. Microfilariae has been considered to be a contributing factor in anaemic processes due to intravascular haemolysis as a result of destructive motility[13] (Ishihara *et al.* 1981 ; Kitagawa, Sasaki, & Ishihara 1989 ; Nielsen *et al.* 2006 ; Ziegler, Käufer-Weiss, & Zahner 1991), and erythrocytes with an already compromised membrane architecture due to oxidative damage (like those in the quokka), may be more susceptible to lysis. However, we did not observe significant differences in the PCV or RBC of microfilariae-positive and microfilariae-negative animals, in both Rottnest Island and mainland groups (see section 7.4.2). It is interesting to note though, that the highest seasonal prevalences (autumn: 51.7%, winter: 45.8%) of microfilariae, matched the lowest average RBC, PCV and HGB values. In light of the similarity of leukocyte parameters of microfilariae-positive and microfilariae-negative animals, that leukogram measures in both groups were below the values reported for morbid quokkas with haematological signs of an inflammatory reaction (Clark 2006), coupled with the absence of left shift or associated morphologies (e.g. Döhle bodies, foamy cytoplasm) in blood smears, an inflammatory response to the

[13] refers to the mechanical intravascular lyses of erythrocytes due to the movement of microfilariae

presence of microfilariae may have not been present at the time of the study. The clinical significance of microfilariae may be difficult to establish as both groups (positive and negative by light microscopy) had similar counts of WBC and EOS. In contrast, microfilaraemia was considered to be mild on the mainland group as 75% (6/8, 95% CI 41-93) of positive animals presented the lowest degree of microfilaraemia (i.e. 1-5 microfilariae per low power field), and there were no differences in RBC, PCV, HGB, WBC and EOS counts, between positive and negative animals either.

As mentioned, erythrogram parameters in the mainland group were significantly higher (except MCV) than those of their conspecifics on Rottnest Island. Erythrocytosis as evidenced by an increased RBC and PCV in the mainland group may have occurred and should be considered when looking at its HMT profile. Although we can not entirely rule out an absolute erythrocytosis either primary or secondary, it is unlikely considering that animals from both subpopulations (i.e. Rottnest Island and mainland) did not present with obvious signs of inadequate tissue oxygenation or severe cardiopulmonary disease, and overall were apparently healthy. Conversely, a transient relative erythrocytosis due to redistribution of red blood cells secondary to splenic contraction is more likely, considering that there were no obvious signs of dehydration or fluid shifts (e.g. hyperthermia) at the time of physical examination. Splenic contraction secondary to stress and pain in small animals has been shown to cause a 30%-60% increase in PCV (Harvey 2012 ; Thrall 2012b). In this study, the PCV of the mainland cohort was 22.5% greater than in the Rottnest Island cohort. Although splenic contraction may in fact have occurred in both groups (Rottnest Island and mainland), mainland animals may have been under greater stress due to longer in-trap times and physical restrain than animals on Rottnest Island, by the time they were anaesthetised (Barnes, Goldizen, & Coleman 2008 ; Harvey 2012), thus having a greater degree of stress-mediated alterations in their complete blood count.

In macropods, NEUT have been reported to be the most abundant leukocyte (Vogelnest & Portas 2010), followed by LYMPH and EOS (Clark 2004), and previous research on *S. brachyurus* in the wild and in captivity also confirm this leukocyte hierarchy (Clark 2006 ; Clark & Spencer 2006). Our results for both Rottnest Island and mainland cohorts are also in agreement with the findings of Clark (2006) and Clark and Spencer (2006). However, in our study mainland animals had significantly lower LYMPH and EOS counts as well as higher NEUT counts than Rottnest Island animals. Naturally occurring differences in leukocyte populations have been previously reported in native Australian marsupials (western ringtail possum (P. occidentalis) Clarke *et al.* 2013 ; e.g. tammar wallaby (M.

eugenii) McKenzie, Deane, & Burnett 2002 ; woylie Pacioni *et al.* 2013), and attributed to factors such as age, climate, nutrition and underlying disease. However, our results are in contrast to the observed differences recorded by Clark and Spencer (2006) in the NEUT, LYMPH and EOS counts between free-ranging quokkas on Bald Island and free-ranging quokkas on the mainland (i.e. near Albany), in which these three parameters were lower in the mainland cohort. These differences may be explained either by a small sample size in the study by Clark and Spencer (2006), or by differences in animal handling protocols, as animals from island and mainland groups in the study of Clark and Spencer (2006) were handled identically, where as in our study, mainland animals might have been affected by stress to a greater degree (longer in-trap times and physical restraint prior to anaesthesia) than animals on Rottnest Island. A stress leukogram in macropods like in other mammals (Cattet *et al.* 2003 ; Harvey 2012 ; Superina & Mera y Sierra 2008 ; Weber *et al.* 2002 ; Weiser 2012), is characterised primarily by a high count of NEUT and low counts of LYMPH and EOS (Clark 2006 ; Vogelnest & Portas 2010); with high MONO counts also reported in the red kangaroo (*M. rufus*) (Clark 2006). With the exception of MONO, the data obtained for the mainland group (i.e. LYMPH, NEUT and EOS counts) seems to be in agreement with previously described stress leukogram profiles. Chronic stress which has not been thoroughly studied in animals, let alone in macropods, has also been associated in humans with leukograms exhibiting neutrophilia, leukopenia and eosinopenia (McKinnon *et al.* 1989), and would rise the question as to whether this stress leukogram in the mainland quokka is a reflection of environmentally related stress, and not exclusively related to trapping and handling. Alternatively, the higher EOS counts observed in the Rottnest Island cohort may reflect a greater exposure to gastrointestinal parasites, while the higher LYMPH counts could potentially be related to a greater degree of antigenic stimulation.

Comparing the reference intervals calculated in this study (see section 7.4.3) for the leukogram component of the Rottnest Island cohort, against the same parameters available in the literature for animals in captivity (Hawkey 1975 ; Lewis, Phillips, & Hann 1968 ; Vogelnest & Portas 2010), generally captive quokkas appeared to have higher means for all parameters (i.e. WBC and differential counts). For instance, WBC and NEUT were two times higher than in our data, with WBC values falling outside our reference intervals (leukocytosis); averages for LYMPH were 3-6 times higher than in our data, and either fall in the upper range or outside of our reference intervals (lymphocytosis). Smaller differences were observed for EOS and MONO. Given that leukocytes are generally affected by multiple factors (Clark 2004), and that lymphocytosis is known to be a feature in many different conditions involving viral, bacterial, parasitic, neoplastic (e.g.

leukaemia), and hormonal conditions (e.g. hypoadrenocorticism), as well as stress, it is unclear as how these differences came to be so marked, and further research would be necessary to determine their cause.

Flower cells (see section 7.4.3, and Figure 7-11a,b,c,d) were observed in peripheral blood of Rottnest Island animals. Although surface markers and special stains would have facilitated the differentiation of these cells from other haematopoietic lineages (Harvey 2012), these resources were not available to this project. Nonetheless, these cells were thought to be of the lymphocyte lineage, due to the appearance of a granular cytoplasm, coarsely clumped chromatin, and their smaller N:C ratio, when compared to MONO, NEUT and EOS (the nucleus is not always easily visible in basophils). Though flower cells appeared to share some features of typical LYMPH[14] in *S. brachyurus*, as described by Clark (2004), their nuclear architecture, appearance of cytoplasm, relatively greater N:C ratio, and overall size were different, which was also the case when comparing flower cells and Atly to previously described reactive LYMPH[15] in *S. brachyurus* and other macropods (Clark 2004). To the best of our knowledge, these cells have not been reported before in *S. brachyurus*, or any other macropod species. In summary, flower cells in blood smears of Rottnest Island animals, were typically bigger than LYMPH, as big or bigger than other leukocytes such as NEUT or BASO, but not bigger than MONO. They had a polylobated nucleus with defined shapes (i.e. flower-like nucleus), a fine and weak granular basophilic cytoplasm, and a higher N:C ratio than LYMPH but smaller than other cells (i.e. MONO, NEUT and EOS). Flower cells and Atly were not considered to exhibit features of programmed cell death (Harvey 2012), as shrinkage (apoptosis) of the cell and fragmentation of the nucleus (karyorrhexis) were not evident.

It could be argued that flower cells and Atly in blood smears of quokkas are normal LYMPH morphologies representing different degrees of immunostimulation, and that these cells were misidentified in the Rottnest Island group, accidentally missed while assessing blood smears of mainland animals, or simply not capture due to a smaller sample size (mainland). However, it seems unlikely considering that blood smears from Rottnest Island and mainland cohorts were evaluated in duplicate and by the same operator, leukocyte differentials were done using 200 cells, blood smears were examined in their entirety, and their morphology does not match that of normal leukocytes in *S. brachyurus* (Clark 2004). Alternatively, the nuclear morphology and overall appearance of flower cells and Atly in *S. brachyurus* may correspond to LYMPH that have lost nuclear

[14] Lymphocytes are small with a round to ovoid nucleus that occasionally present an indentation or are deeply *cleaved*, their chromatin appears coarsely clumped and have a small rim of basophilic cytoplasm (Clark 2004)
[15] Intense basophilic cytoplasm, indented or deeply cleaved nucleus and a small N:C ratio (Clark 2004)

integrity due to a neoplastic alteration. Pleomorphic lymphocytes with a nucleus that varies in shape from a single indentation to a complex convoluted appearance are often observed in lymphoproliferative disorders in other species (Harvey 2012). Flower cells were present in 23 of 108 Rottnest Island animals, ranging from 1-6 cells per 200 leukocytes. Interestingly, WBC counts were significantly (p= 0.026) higher in flower cell-positive animals than in those that were negative. However, whether these high counts were attributable to the presence of flower cells and Atly only, remains unknown.

Proliferative disorders of hematopoietic nature have been reported in the whiptail wallaby (*Macropus parryi*), agile wallaby (*Macropus agilis*), common wombat (*Vombatus ursinus*), sugar glider (*Petaurus breviceps*) (Canfield, Hartley, & Reddacliff 1990a), northern quoll (*Dasyurus hallucatus*), fat-tailed antechinus (*Pseudoanlechinus macdonellensis*) (Canfield, Hartley, & Reddacliff 1990b), *Antechinus* sp. (Attwood & Woolley 1973), as well as in pademelons (*Thylogale* spp.) and the Tasmanian bettong (*Bettongia gaimardi*) (Vogelnest & Portas 2010), however, leukocyte morphology has not been reliably and comprehensively recorded. Atypical lymphocytes have been described in peripheral blood of koalas infected with *Koala retrovirus* (KoRV) an endogenous type C gammaretrovirus (Connolly *et al.* 1998 ; Spencer & Canfield 1996), with nuclear shapes described as indented or folded, convoluted or lobulated (Tarlinton *et al.* 2005), but the nuclear architecture differs greatly from that of flower cells in peripheral blood of quokkas on Rottnest Island. However, LYMPH with similar atypical morphology (see Figure 7-11e), have been observed in peripheral blood of asymptomatic human carriers of human T-cell leukaemia virus type 1 (HTLV-1, a deltaretrovirus) (de Oliveira *et al.* 2010). To our knowledge, these cells have not been reported in healthy HTLV-1 uninfected individuals or in individuals with other clinical conditions different to HTLV-1 infection (Shimoyama *et al.* 1983). The unique nuclear morphology of flower cells in humans is believed to be the result of microtubule rearrangement of the cytoskeleton as a consequence of alteration in the phosphatidyl-inositol 3-kinase (PI3-kinase) cascade (Fukuda *et al.* 2005). , flower cells have been observed in 7% of HTLV-1 carriers (Sacher *et al.* 1999), and in more than 50% of patients with adult T-cell leukaemia/lymphoma (ATL) (Fukuda *et al.* 2005). Some authors consider that the presence of these cells (i.e. flower cells and Atly) in asymptomatic carriers of HTLV-1 is a risk factor for developing ATL (Tachibana *et al.* 1992 ; Yamaguchi *et al.* 1988), while others believe these cells represent a preleukaemic event (Shimoyama 1991). More recently, flower cells and prototype cells (i.e. Atly) (see Figure 7-11f) have been recorded in mice five months after being infected with sublethally irradiated HTLV-1 producing cells (Tezuka *et al.* 2014).

To rule out the presence of a deltaretrovirus in animals with flower cells in peripheral blood, blood samples were screened for the presence of retroviral DNA using three different approaches, a pan-retroviral primer method targeting the polymerase gene (Donehower *et al.* 1990), and two pan-deltaretroviral specific primer sets targeting highly conserved regions of the deltaretroviral genome (Burmeister *et al.* 2007 ; Burmeister, Schwartz, & Thiel 2001). Unfortunately, conclusive results were not obtained. The unknown nature and significance of flower cells in peripheral blood of quokkas on Rottnest Island, warrants further investigation, especially when cells with similar morphology have been associated with HTLV-1 infection in humans. Additional tests such as immunophenotyping and bone marrow examination in addition to molecular screening of retroviruses should be considered, ideally in a capture-recapture study framework in order to observe the progression of these cells across time.

Plasma chemistry analytes in *S. brachyurus* were generally similar to previously reported data for other free-ranging macropods (Barnes, Goldizen, & Coleman 2008 ; McKenzie, Deane, & Burnett 2002 ; Stirrat 2003), as well as in captivity (Vogelnest & Portas 2010). However, in comparison with the only plasma chemistry data available for *S. brachyurus* today (Teare 2002) which originates from captive individuals, our data varied considerably, with both subpopulations (Rottnest Island and mainland) presenting higher values for ALP, ALT, AST, CK, BILT, PROT, UREA, and GLOB, some times by two-fold or more; and lower values (though not as different), for GLUC, CALC, ALB and CHOL. In contrast, CREAT and PHOSP were lower in Rottnest Island animals and higher in mainland animals, than the reported values in captivity. Differences in plasma chemistry profiles between free-ranging and captive subpopulations are not uncommon, as this has been previously reported in other marsupials species, for instance in the western ringtail possum (*Pseudocheirus occidentalis*) (Clarke *et al.* 2013), the Gilbert's potoroo (Vaughan *et al.* 2009), and the western barred bandicoot (*Perameles bougainville*) (Bennett *et al.* 2008). Generally, these studies attributed these differences to nutritional factors, as well as stress particularly in relation to CK and AST. In our case, we also consider that environmental factors such as the absence of seasonal effect in captive groups, as well as nutritional differences, may account for most of the differences (including vit. E) between free-ranging quokkas (this study) and that of captive animals (i.e. ISIS), with stress playing a secondary role (e.g. familiarity with handling). However, any direct comparison between these datasets should be done cautiously considering the small sample size (i.e. 2-4 animals) used in the ISIS data.

A comparison between Rottnest Island and mainland data, revealed significant differences in their blood chemistry profiles, with CK, ALT, ALP, AST and vit. E representing the greatest percentage of contribution to that difference. Although higher levels of the aminotransferases, AST and ALT, in conjunction with higher levels of ALP and GGT may indicate hepatic injury and insufficiency (Tennant & Center 2008), this may not be the case considering that other laboratory findings (e.g. low GLUC, high BILT, increased WBC) and clinical signs (e.g. ascites, icterus), that are frequently (but not always) found concomitantly in this condition, were not present in the mainland sample. Rather, higher levels of AST and CK may have originated from muscle as it has been proposed previously, particularly associated with prolonged in-trap times as well as extended and stressful physical restraint (Clarke *et al.* 2013 ; Vaughan *et al.* 2009). Although ALT has its highest activity in liver, high levels of this transaminase in plasma can also be associated with muscle injury (Tennant & Center 2008). The exceedingly high levels of ALP in *S. brachyurus* from Rottnest Island (see Table 7-9), were not just higher than any other available data for macropods (Barnes, Goldizen, & Coleman 2008 ; McKenzie, Deane, & Burnett 2002 ; Vogelnest & Portas 2010), but were at least 75% above the highest value reported in macropods (Swamp wallaby $\bar{x}$=3,306, SD±1,071) and were at least doubled when compared to mainland animals. ALP is an enzyme with nonspecific activity, with tissue isoenzymes known to be present in liver, bone, kidney, intestine and placenta, however, in domestic animals it is believed that plasma ALP originates primarily from liver and bone (Allison 2012 ; Tennant & Center 2008). Its increased activity is particularly associated with liver damage due to cholestasis (impaired bile flow) and increased osteoblastic activity particularly in young animals. Cholestasis in Rottnest Island animals is unlikely, considering there was no remarkable increase in GGT which was found to be within the range reported for other apparently healthy macropod species (i.e. *M. giganteus*, *M. fuliginosus*) (Vogelnest & Portas 2010). Furthermore, common signs of hepatic hyperbilirubinemia (e.g. icterus) which usually accompany cholestasis, were not present. Some studies in other marsupial species have found higher levels of ALP in young individuals compared to adults (Barnes, Goldizen, & Coleman 2008 ; McKenzie, Deane, & Burnett 2002 ; Vaughan *et al.* 2009) which were attributed to increased osteoblastic activity. This may not be the case in this study, given that most of the Rottnest Island animals were considered to be adults. ALP in plasma can also increase due to the activity of the corticosteroid isoform (CALP) in response to persistent levels of endogenous corticosteroids during chronic stress (Allison 2012). However, this isoform has only been detected in dogs (Celia Schultz *pers*. *comm*. 2015). These high levels of ALP in plasma are well known for quokkas in captivity (Tim Portas *pers*. *comm*. 2013), and it appears to be the same in free-ranging individuals. Although CALP has not been studied in macropods,

chronic stress appears as a possible pathway for these high levels, otherwise this could simply be part of *S. brachyurus* normal physiology. To answer this question, tissue enzyme studies of all major organs, as well as skeletal muscle and intestine, of apparently healthy quokkas would be necessary.

Despite considerable research on muscle dystrophy of nutritional origin in *S. brachyurus* (Kakulas 1961 ; Kakulas 1963a, 1963b ; Kakulas 1983 ; Kakulas & Adams 1966) showing that the condition was not only triggered by vit. E (alpha-tocopherol) deficiency (Kakulas 1961) but also by small enclosures (Kakulas 1963a), and that clinical signs of muscle dystrophy such as weight loss, progressive wasting of hindlimb muscles and paralysis were also successfully reversed with the supplementation of vit. E at a dose of 200-600 mg daily (Kakulas 1963b ; Kakulas 1983), it is surprising that reference levels for vit. E in plasma of *S. brachyurus* were never determined. The only available data today, are that of Vogelnest and Portas (2010), where apparently healthy *S. brachyurus* (n= 5) at Taronga Zoo had vit. E levels in plasma ranging from 2.9-3.2 mg/L, while at the Melbourne Zoo (n= 4) levels were slightly lower ranging from 2.0-2.6 mg/L. We determined higher levels of vit. E in mainland animals ($\bar{x}$= 9.92 mg/L SD± 2.85, Reference Interval 5.23-16.5 mg/L) than in RI animals ($\bar{x}$= 6.65 mg/L SD± 1.81, Reference Interval 4.03-10.5 mg/L). This difference could be explained by better diets (greater content of vit. E) on the mainland than on Rottnest Island. In fact, although not drastic, lower values of vit. E in plasma were observed on Rottnest Island during the dry months of the year, time in which diets not just decreased in amount but in quality. The values given by Vogelnest and Portas (2010) for captive individuals, are below our findings for apparently healthy free-ranging animals, which makes us believe that captive animals are not receiving the necessary supplementation of vit. E, and may be at risk of oxidative degradation of membrane phospholipids, as well as disruption of other critical cellular processes including transduction pathways such as the phosphatidylinositol 3-kinase responsible for cell growth and proliferation (Rucker, Morris, & Fascetti 2008), which in turn is involved in proliferative disorders (Fukuda *et al.* 2005). According to the concentrations of vit. E in plasma that we obtained for free-ranging animals on Rottnest Island, we believe it is advisable to supplement vit. E so as to reach plasma concentrations within 5.23 mg/L and 16.5 mg/L.

To our knowledge, this study provides the first and most comprehensive haematology and blood chemistry reference intervals for free-ranging quokkas in WA. Our data have shown that there are significant differences between the haematology and blood chemistry profiles of the subpopulations studied (Rottnest Island and selected locations on the

mainland: Jarrahdale, Collie and Walpole), and highlights the importance of establishing reference intervals not just for the species as a whole but for individual subpopulations, given the uniqueness of their environmental, parasitic and nutritional conditions among others. We believe that a comparative case between our data and that of ISIS (Teare 2002) would not be informative because ISIS data is limited by sample size (2-4 animals) and the absence of other relevant information (e.g. nutrition, environmental conditions). We therefore advise against updating ISIS with our data, but instead suggest updating ISIS database by increasing the sample size. Considering that our data reflects free-ranging conditions, and these conditions are for the most part not present in captive populations, we also advice against using our data to manage *S. brachyurus* in captivity. We believe that the presence of LYMPH with similar nuclear architecture and overall appearance (i.e. flower cells and Atly) in other species with deltaretroviral infection, presents as imperative, the need to carry out further studies to establish the meaning and significance of flower cells and Atly in peripheral blood of members of the biggest extant subpopulation of *S. brachyurus*. We believe our work can facilitate the management of *S. brachyurus* in the wild by providing baseline data that can be used to monitor the health status of these subpopulations. This is especially important as these animals live in a rapidly changing environment (e.g. climate change, infectious diseases, reduction of habitat). We echo the other voices advocating that disease and health surveillance should be ongoing efforts in native species management plans. This would allow for the development of contingency plans that would assist relevant management bodies to respond rapidly to new disease events.

8. General Discussion and Synthesis

8.1 Introduction

The quokka, *Setonix brachyurus* (Quoy and Gaimard 1830) (Marsupialia: Macropodidae), is a small wallaby, and the only member of the genus *Setonix* (Hume *et al.* 1989). It is endemic to the mainland of south-west WA and two offshore islands: Rottnest Island and Bald Island (de Tores *et al.* 2007), and is an icon to the Western Australian (WA) culture. However, the species has suffered a marked contraction in its geographical range since European settlement (Department of Environment and Conservation 2013), with a drastic decline observed in the 1930s (Department of Environment and Conservation 2013 ; White 1952), for which the red fox (*Vulpes vulpes*) is believed to be the most important factor (de Tores *et al.* 2007). Today, the species persists in heavily fragmented subpopulations and faces threats such as predation (de Tores *et al.* 2007), habitat clearing (Gole 2006), climate change (Gibson *et al.* 2010), and a possible continuing decline in area of occupancy and extent of occurrence. Consequently, the species is considered as threatened fauna, at a local, national and international level (de Tores *et al.* 2008 ; de Tores *et al.* 2007). The species has been extensively studied, and there is a reasonable amount known about its ecology (Blumstein, Daniel, & McLean 2001 ; Dunnet 1962, 1963 ; Hayward 2002 ; Hayward 2005 ; Hayward *et al.* 2003 ; Niven 1970 ; Poole *et al.* 2014 ; Shield 1958 ; White 1952), biology (Sadleir 1959 ; Sinclair 1998), nutrition (Hayward 2005 ; Poole *et al.* 2014), physiology (Bartholomew 1956 ; Fleming, Harman, & Beazley 1996 ; Makanya, Haenni, & Burri 2003 ; Makanya *et al.* 2001 ; Makanya *et al.* 2007), and behaviour (Bonney & Wynne 2004 ; Wynne & Leguet 2004). However, the study of its health (free-ranging) and agents of disease is limited.

Although research on the diseases of the quokka has been largely ignored, there are anecdotal records indicating that at least six disease outbreaks were associated with mass mortality of individuals. These were recorded between 1901 and 1931, a time period that pre-dates the arrival of the red fox in WA. Unfortunately, these disease outbreaks were not investigated. There is mounting evidence from the last three decades that indicates that disease can have devastating impacts on wildlife populations (Botero *et al.* 2013 ; Cohn 2008 ; Daszak & Cunningham 1999 ; Daszak, Cunningham, & Hyatt 2000 ; Gog, Woodroffe, & Swinton 2002 ; Hawkins *et al.* 2006 ; Heard *et al.* 2013 ; Hess 1994 ; Jones *et al.* 1997 ; Leendertz *et al.* 2006 ; McCallum & Dobson 1995 ; Pedersen *et al.* 2007 ;

Schloegel *et al.* 2006 ; Scott 1988 ; Smith, Sax, & Lafferty 2006 ; Vaughan 2008 ; Woolford *et al.* 2009 ; Wyatt *et al.* 2008), however, little is known of the diseases and agents of disease that occur in free-ranging quokka, and only a limited number of studies have been carried out (Austen *et al.* 2014 ; Austen *et al.* 2009 ; Austen *et al.* 2011 ; Clark 2006 ; Clark & Spencer 2006 ; Clark & Spencer 2007 ; Dickson & McNeice 1982 ; Johansen *et al.* 2005 ; Wilks, Kefford, & Callinan 1981 ; Yen 1983); with the majority of these being focused on the ecology of *Salmonella* spp. infections on Rottnest Island (Hart 1980 ; Hart, Bradshaw, & Iveson 1985, 1986 ; Hart, Iveson, & Bradshaw 1987 ; Hart *et al.* 1982 ; Iveson 1977 ; Iveson & Bradshaw 1973 ; Iveson, Bradshaw, & Hart 2007 ; Iveson & Hart 1983). These studies had little or no comparison of different subpopulations, used small sample sizes, mostly studied animals in captivity, and had little or no assessment of the possible associations between the presence of an infectious agent and the health of the animal.

In response to the dearth of knowledge in quokka health, this thesis not only provides the most comprehensive review of diseases and infectious agents reported in the species since the early 1900s, but explores the presence of selected infectious agents in multiple subpopulations, and the possible associations between these agents and the health status of the animals at the time of sampling. Similarly, this thesis provides epidemiologically significant data, such as the prevalence and characterisation of the infectious agents detected. Finally, this thesis provides the most complete set of physiologic reference intervals for haematology and blood chemistry parameters in apparently healthy *S. brachyurus*. The results of this research are significant in the areas of conservation medicine, wildlife management, and public health.

8.2 Thesis summary

An extensive literature review on the current conservation status of the quokka, its known disease conditions and the detected infectious agents, was carried out by accessing and collating information from local, national and international government and non-government databases. Captive population numbers and their location were updated with data from the Zoo and Aquarium Association for Australasia, as well as through direct contact with local projects indicated by the Western Australian Department of Parks and Wildlife as having animals in captivity at the time of the study. The disease section is accompanied by general concepts of the infectious organisms involved such as taxonomy, transmission and pathogenesis, as well as a thorough description of the reported quokka clinical cases, when available. These data were further enriched with information from the Australian Registry of Wildlife Health pathology database, which contains clinical

cases reported by Australian institutions with ex-situ conservation programs; cases that usually do not reach mainstream science publications.

The experimental aspect of this thesis focused on obtaining valuable epidemiological data (e.g. presence/absence, prevalence, distribution, relation with concomitant infections, and influence of factors such as sex, season and location) for *Salmonella* spp., *Cryptococcus* spp., and MaHV-6 (a novel macropodid herpesvirus); and assessing their impact on the health [e.g. associations with physical examination findings, and effect on haematology (HMT) and blood chemistry (BLC) parameters, as well as peripheral blood cell morphologies (PBCM)] of the quokka. This was coupled with a comprehensive analysis of their HMT and BLC parameters to construct reference intervals for apparently healthy animals. Comparisons were made between subpopulations. Additional data was obtained for intraerythrocytic parasites (i.e. *Theileria* sp. and *Babesia* sp.), trypanosomes, microfilariae, and saprophyte fungi.

To accomplish these outcomes, over 150 animals were captured between Rottnest Island and three mainland locations: Jarrahdale, Collie and Walpole. Animals were anaesthetised, physically examined, and biological samples collected that included faeces, nasal swabs and blood. The diagnostic methodologies used in this research allowed the detection and characterisation of the target organisms. Culturing, serotyping and/or DNA sequencing were used for *Salmonella* spp. and *Cryptococcus* spp., while PCR, DNA sequencing and phylogenetics were used to detect and identify MaHV-6, *Theileria* sp., *Babesia* sp. and trypanosomes. Due to the sample size handled in this project, a robust non-parametric approach was used to construct reference intervals for HMT and BLC, as recommended by the International Federation of Clinical Chemistry and Clinical and Laboratory Standards Institute. These reference intervals were calculated for males and females on both locations: Rottnest Island and mainland. This research project successfully grew, stored and established a bank of live organisms recovered from quokkas on Rottnest Island and the mainland of WA. These included *Salmonella* serovars, mostly of the *enterica* group, *C. neoformans* var. *grubii* and *C. magnus*, as well as other yeasts. Likewise, a bank of blood samples has also been established, along with a collection of more than 300 blood smears. All these materials and samples have significant value for future and retrospective studies. Lastly, as part of this chapter, and in an attempt to address an examiner's request, an attempt to explore and visualise the interrelations of concomitant infections with HMT and BLC parameters of infected animals, was made (see below).

8.3 Health and disease status

Quokkas on Rottnest Island and on the mainland of WA that this project studied were considered to be apparently healthy at the time of sampling. This is supported by the absence of obvious signs of disease during examination, as well as the absence of statistical effect of the various organisms studied, on the HMT, BLC and PBCM of the animals.

8.3.1 *Salmonella infections*

Key epidemiological characteristics of *Salmonella* infections in the Rottnest Island quokka, that were first described in the late 70s and early 1980s (Hart 1980 ; Hart, Bradshaw, & Iveson 1985 ; Hart *et al.* 1982 ; Iveson & Bradshaw 1973 ; Iveson & Hart 1983), appear to remain unchanged. Data supporting this includes a similar prevalence of *Salmonella* to that published in previous studies (Iveson, Bradshaw, & Hart 2007 ; Iveson & Hart 1983), a seasonal pattern with a greater prevalence of *Salmonella*-positive cases in summer than in winter, and a lower prevalence of *Salmonella*-positive cases in populated areas of the island. This absence of change in the basic aspects of the ecological relation between *Salmonella* and quokkas may reflect stability in the host-parasite-environment complex on Rottnest Island. However, it is possible that this complex is indeed changing and may be more intricate than once thought. For example, we have provided evidence of a strong association between a previously-identified microfilariae infection (Yen 1983), and *Salmonella* spp. infections in the Rottnest Island quokka, whereby animals positive to microfilariae were more likely to be infected with *Salmonella* spp., there was only weak evidence that *Salmonella* does *not* have an effect on the HMT and BLC of quokkas, and finally, four new *Salmonella* serovars were isolated (*S. enterica* subsp. *enterica*= 3, *S. enterica* subsp. diarizonae= 1). Whether these findings and interactions started to occur recently or not, is difficult to establish when disease surveillance has not been carried out previously.

Historically, *Salmonella* infections have been attributed for the most part to nutritional stress (Hart, Bradshaw, & Iveson 1985), however, the strong and significant association ($p< 0.05$) between microfilariae and *Salmonella* obtained in this study (see Section 4.3.4), where microfilariae-positive animals were 3.88 (CI 1.31-11.5) more likely of being positive to *Salmonella* than microfilariae-negative animals, may suggest that concomitant infection with microfilariae is a predisposing factor for *Salmonella* infections in Rottnest Island quokkas. However, further research would be necessary to obtain proof of

causality. Both organisms were found to be associated with lower WBC counts ($p < 0.04$), which suggest that both may be involved in some degree of immunosuppression. However, filarial nematodes are known for exerting a potent downregulating effect on the immune system of the infected host (Moore *et al.* 1993 ; O'Brien *et al.* 1979 ; O'Regan *et al.* 2014 ; Ochsenbein *et al.* 1999 ; Semnani *et al.* 2008 ; Su *et al.* 2014 2666).

Previous studies on *Salmonella* infections in Rottnest Island quokkas suggested that *Salmonella* did not cause disease (Hart 1980 ; Hart, Bradshaw, & Iveson 1985 ; Hart *et al.* 1982 ; Iveson & Bradshaw 1973 ; Iveson & Hart 1983), and there were no negative effects of the bacterial infection on the physiology of the animals. However, there appear to be some evidence that infection with *Salmonella* spp. is having some effect on the organic state of the animals. More specifically, when compared to negative animals, positive animals had lower values of RBC, PCV, HGB, WBC, LYMPH, NEUT, EOS, and BASO, while MONO were lower in negative quokkas. Blood chemistry profiles were also different, with positive animals having higher values for ALP, BILT, CREAT and UREA, and lower values for GLUC, PROT, and ALB, than those showed by negative animals. Although these differences were found to be not significant by PERMANOVA analyses (i.e. $p > 0.05$), the p values obtained for haematology and blood chemistry, that ranged between 0.05 and 0.08 (depending on the covariate used: sex or season), were considered as insufficient evidence of the absence of effect of *Salmonella* spp. infection on the physiology of the host. In other words, these results could be considered as being suspicious of an active salmonellosis, and not simply a carrier asymptomatic stage. However, there is not enough evidence to support this.

It is impossible to predict whether or not *Salmonella* infections could become a threatening process for the Rottnest Island quokka at some point in the near future. However, it seems possible that downregulation of the immune system caused by increasing stressors (e.g. changes in climate, loss of habitat and concomitant disease such as microfilaria infection) could lead to widespread clinical salmonellosis.

The prevalence (4.8%) and the serovar (*S. enterica* subsp. *diarizonae* (IIIb) ser. 50:k:z35) detected in mainland quokkas are worth noting. Negative results for *S. enterica* subsp. *enterica* serovars from mainland samples suggest that the recovery of *S. enterica* ser. Muenchen and *S. enterica* ser. Newington (the only serovars recovered) from mainland quokkas in previous studies (Hart 1980 ; Hart, Bradshaw, & Iveson 1986), was in fact the result of cross contamination with trapping equipment previously used on Rottnest Island, as the authors proposed. Serovars of the *Salmonella enterica* subsp. *enterica* group are

more pathogenic than serovars of the other five *Salmonella* groups (i.e. II, IIIa, IIIb, IV and V), and if animals on the mainland are immunologically naïve, this would explain the mortalities that Hart (1980) reported, with quokkas dying acutely some time after being in contact with the contaminated equipment. Alternatively, the negative results for *Salmonella enterica* subsp. *enterica* in mainland quokkas obtained by this study, may be attributable to host immunity, in which case, the peracute mortalities reported by Hart (1980), may have been caused by either an infectious agent other then *Salmonella* spp., or by a different non-infectious disease process.

8.3.2 *Cryptococcus neoformans var. grubii and C. magnus infections*

Previously, the only evidence of exposure to *Cryptococcus* spp. (particularly *C.* gattii but not only) in the quokka was for captive animals, where *Cryptococcus* spp. have been considered to be agents of both primary and secondary (or incidental) disease (ARWH archive records 1999-2014; Krockenberger *et al.* 2005 ; Vaughan 2008 ; Vogelnest & Portas 2010). It has also previously been reported that quokkas can be asymptomatic carriers, where animals with secondary disease did not show signs of disease. This study provides evidence that exposure to highly pathogenic *Cryptococcus* spp. occurs in wild quokkas, and that infection with *C. neoformans* var. *grubii* and *C. magnus* can be asymptomatic, with positive animals showing no detectable signs of clinical disease at the time of examination, supported also by the absence of significant differences in the HMT, BLC, and PBCM indicators.

Despite the absence of clinical disease in Rottnest Island animals positive to *C. neoformans* var. *grubii*, the isolation of this yeast is significant for both animal management and public health. *C. neoformans* var. *grubii* can form silent cryptococcal foci in the respiratory tract (Merchant & Packer 1967 p. 549-566), that under the right conditions (e.g. immunosuppression) can reactivate and become the source of fatal systemic disease (Garcia-Hermoso, Janbon, & Dromer 1999 ; also with C. gattii see Illnait-Zaragozi *et al.* 2011 ; Krockenberger *et al.* 2002). All captive individuals across Australian zoos (ex-situ) were sourced from Rottnest Island (mostly from settled areas due to easier trapping), and it is likely that a proportion of these animals therefore would have arrived at their collections with pulmonary cryptococcal foci and were at a greater risk of developing clinical disease than previously thought. Disease due to *C. neoformans* var. *grubii* is likely to occur in Rottnest Island quokkas (in-situ), for which two main events must be present: immunosuppression of the host, and exposure to cryptococcal organisms as a result of environmental contamination. Immunosuppression is likely to occur under rapid changes

in environmental pressures, particularly if these pressures unfold faster than the quokka can adapt to. Whether greater exposure to the organism does or does not occur, and to what degree, can only be explored when the environmental source or sources for *C. neoformans* var. *grubii* are identified.

Immunosuppression and exposure to a high number of infectious cryptococcal organisms are also two important factors to consider from a public health perspective, given that these two factors increase the risk of disease in humans. Consequently, institutions responsible for public health and the Rottnest Island Authority are advised to considered exposure to *C. neoformans* var. *grubii* by people as likely. Although it is generally accepted that cryptococcosis is not a zoonotic disease, unless the relevant studies are carried out, direct transmission from quokkas to humans can not be rule out. Unless the environmental source(s) are determined, preventative measures and response protocols could not be implemented.

At present, *C. neoformans* var. *grubii* infection does not appear to have a negative impact on the health of quokkas on Rottnest Island, determined by the absence of obvious clinical signs of disease, and no effect of *C. neoformans* var. *grubii* on the HMT, BLC and PBCM of positive animals. However, clinical disease (cryptococcosis) is likely to occur at some point in time. The risk of disease will increase according to the degree of environmental contamination, the degree of exposure to that environmental source(s), and the degree of immunosuppression of the host. These factors would play a similar role in both, animals and people alike. Whether this disease could present as a threatening process to the quokka in the future, it is possible but unlikely. This is particularly because the organism is not known to be transmitted from animal to animal, which would reduce the risk of a widespread outbreak due to direct contact. Additionally, *C. neoformans* var. *grubii* is known to be restricted to certain environmental sources typically found in settled areas, which according to the results of this study, would appear to be also the case on Rottnest Island. These limitations (i.e. source and location) would theoretically restrict exposure to only animals in those areas. Furthermore, quokkas on Rottnest Island are not known to move a great deal across the island, therefore limiting the groups that could come in contact with the environmental source or sources for *C. neoformans* var. *grubii*.

8.3.3 *Macropodid herpesvirus 6*

A fatal case of a captive quokka at the Melbourne Zoo that succumbed to infection with *Macropodid herpesvirus* 2 (MaHV-2) (Callinan & Kefford 1981 ; Johnson & Whalley 1987 ;

Johnson *et al.* 1985 ; Wilks, Kefford, & Callinan 1981), was the beginning of a long standing hypothesis that quokkas were not the natural host for MaHV-2. However, free-ranging quokkas were never tested for antibodies against MaHV-2 (or MaHV-1), until now. This study tested over 100 animals across multiple subpopulations (covering at least 80% of the current geographical range of the species) and confirmed that the quokka is not the natural host for MaHV-2 or MaHV-1, given that seropositivity to MaHV-1 and MaHV-2 was only detected in one mainland animal. If quokkas were the natural host for these two viruses, a greater prevalence and distribution would have been seen. Consequently, these results help close an important chapter in the epidemiology of herpesviruses in macropods. The seropositive animal (i.e. to both MaHV-1 and MaHV-2) detected on the mainland may indicate that these two viruses have circulated within the natural range of quokkas on the mainland, and that the natural host of MaHV-1 and MaHV-2 may share the same ecological niche as the quokka.

Through PCR, sequencing and phylogenetic analysis, a novel gammaherpesvirus, tentatively identified as Macropodid herpesvirus 6 (MaHV-6), was detected in apparently healthy quokkas from all major subpopulations where animals were trapped. These animals showed no evidence of disease based on HMT, BLC and PBCM datasets. The absence of obvious disease and the widespread presence of MaHV-6 strongly suggest that the quokka is the natural host for MaHV-6. Its prevalence in WA may be greater than this study determined, considering that two subpopulations (the southernmost ones) were not sampled due to logistic limitations, and that sample sizes for some mainland sites were small.

Gammaherpesviruses are known to remain within the host LYMPH in latent form, avoid lytic replication and rarely cause disease; this seems to explain the asymptomatic presentation of MaHV-6 in the quokka. However, such latency can be interrupted in the natural host through immunosuppression caused by concomitant disease, or from long term exposure to endogenous glucocorticoids due to chronic stress. Chronic stress may be expected in quokkas on both Rottnest Island and the mainland due to various environmental challenges such as land clearing, loss of vegetation to disease, changes in weather patterns, agriculture, predation pressure from invasive animals, and the never ending expansion of human settlements. The clinical presentation of MaHV-6 infection remains unknown. However, although there is no evidence at this point in time, skin lesions (e.g. oral and cloacal ulcers), respiratory signs, and ocular and nasal discharges may be expected. These clinical signs have been previously observed in animals with

clinical disease from all previous characterised macropodid herpesviruses (i.e. MaHV-1, -2, -3, and -4), except MaHV-5.

Even though direct contact (animal to animal) would allow MaHV-6 to readily spread within a mob or subpopulation, predicting the overall impact within that subpopulation is not possible, as many aspects of the pathophysiology of MaHV-6 still need to be understood. Conversely, if spill over from quokkas to other macropods were to occur, the effect of MaHV-6 infection in other species may be more serious, given that herpesvirus infections are usually fatal for species other than the natural host. In any case, continuous surveillance of MaHV-6 in the context of health (i.e. screening for MaHV-6 coupled with a thorough and systematic physical examination) is desirable.

8.3.4 ***Haematology and Blood chemistry***

8.3.4.1 Reference Intervals

Generally speaking, most of the variables measured for both HMT and BLC were within previously reported values for apparently healthy macropods. However, there were significant differences between the haematology and blood chemistry profiles of quokkas on Rottnest Island and on the mainland (section 7.4.1). These site differences are likely the result for the most part, of the interplay of multiple factors such as nutrition, water availability, climate, concomitant disease, presence/absence of predators. However, stress may have also played a role. Animal handling and anaesthetic protocols were the same for all trapping sites, however, in-trap times for mainland animals were longer than those of animals on Rottnest Island. Additionally, animals on the northern sites (mainland) were subjected to longer physical restraint and semi-invasive procedures prior to being anaesthetised. Thus, it is likely that Rottnest Island and mainland animals were exposed to different degrees of stress and therefore had different levels of endogenous glucocorticoids. Whether stress played a significant role in the differences observed between the HMT and BLC of Rottnest Island and mainland animals, is unknown. However, it is a factor to take into account when considering using the calculated reference intervals. Nonetheless, for most parts, the blood parameters suggest greater physiological challenge experienced by Rottnest Island animals.

A number of restrictions should be applied to the use of the reference intervals presented in this work. First, it is advisable that each reference intervals dataset is used for its corresponding subpopulation, considering that physiological parameters are unique to their host-parasite-environment relation. Second, though these datasets can be used with

animals in captivity, it is desirable not to, given the absolute differences between the environmental and dietary conditions among other factors, of wild and captive quokkas. The reference intervals calculated and presented in this project, are not standalone sets of diagnostic data, and should be accompanied by other disease diagnostic techniques (e.g. physical examinations), and careful consideration of external factors (e.g. climate, time of the year, diet).

Particularly significant observations recorded on quokkas in Rottnest Island, are revisited in this section. These include a normocytic hypochromic regenerative anaemia, possibly haemolytic and intravascular in nature; the presence of neoplastic-looking lymphocytes; and low concentrations of vitamin E in plasma (compared to mainland animals).

8.3.4.2 Anaemia

The anaemic state of quokkas on Rottnest Island is most likely the result of multiple factors having a synergistic effect on their haematology. Despite the absence of conclusive evidence for causation, some of the findings observed in this project may partly explain the anaemic state of quokkas on Rottnest Island, and are worth mentioning.

Factors responsible for anaemia may include oxidative stress (free radical reactions), mechanical lysis of red blood cells, and blood loss. Anaemia in quokkas on Rottnest Island has been attributed to a protein-deficient diet (Barker *et al.* 1974 ; Shield 1971), and although dietary studies were not carried out in this project, abundance of Heinz bodies (oxidised haemoglobin) in the erythrocyte of quokkas on Rottnest Island (compared to mainland animals) may have been an indication of high levels of oxidative stress. Protein deficiency may be a possible pathway for triggering the formation of Heinz bodies, with protein-deficient diets involved in decreased activity of at least three erythrocytic enzymes that protect the cell from oxidative injury (Huang & Fwu 1993).

Vitamin E levels in plasma were at least 3 mg/L lower in Rottnest Island animals when compared to mainland animals. Further oxidative damage could be due to the low availability of vitamin E, a potent antioxidant that blocks oxidising reactions, hence preventing the propagation of reactive oxygen species (free radicals) (Brigelius-Flohé & Traber 1999). Low concentrations of circulating vitamin E in plasma have been suggested as a possible aetiology for haemolytic anaemia in other species (Brigelius-Flohé & Traber 1999 ; Dierenfeld, du Toit, & Miller 1988 ; Rucker, Morris, & Fascetti 2008 pp. 706-709) and has been linked to increased susceptibility to peroxide-induced haemolysis (Simon *et*

al. 1998), and diminished erythrocyte life span (Farrell *et al.* 1977 ; Losowsky & Leonard 1967). As a result of the oxidation of membrane lipids, the erythrocyte becomes more fragile and prone to lysis.

Haemolysis could be further enhanced by the presence of intraerythrocytic parasites (i.e. *Babesia* spp. and *Theileria* spp.) that are known to cause physical damage to the erythrocyte (Clark 2004 ; Harvey 2012, p. 102; Thrall 2012d), and by the presence of circulating microfilariae (i.e. genus *Breinlia* sp.) that could cause erythrocyte lysis through destructive motility (Ishihara *et al.* 1981 ; Kitagawa, Sasaki, & Ishihara 1989 ; Nielsen *et al.* 2006 ; Ziegler, Käufer-Weiss, & Zahner 1991). Similarly, inflammatory processes in response to infection could also contribute to the anaemic state of the animals.

Of these factors, *Theileria* sp. and microfilariae are the only two evident in mainland animals. On the other hand, oxidative injury due to low dietary protein and vitamin E content, may not be present due to a greater variety and quality in their diets, while the negative results for *Salmonella enterica* serovars for mainland animals could mean that blood loss may also be absent. However, increasing changes in climate, fire regimes, and land usage can affect the current host-parasite balance (i.e. *Theileria* sp. and microfilariae), as well as food availability, potentially increasing risk of oxidative injury to the erythrocyte and tissues due to diet. Similarly, the risk of exposure to *Salmonella* spp. for mainland animals would likely increase through processes such as increased urbanisation and agriculture.

Anaemia is likely to decrease overall animal fitness requiring: i) reallocation of energy from other processes such as immunity, reproduction and growth to regenerative bone marrow activity, ii) an increase of the overall metabolic rate at the expense of other physiological processes; iii) various degrees of impaired oxygenation of tissues; and iv) a possible shift in the metabolism of the animal to an anaerobic glycolysis, which in turn would favour a drop in the pH that could then cause cell injury by altering the chemical gradient across cell membranes.

8.3.4.3 Flower cells and Atly

Two morphologically unique lymphocytes were evident in quokkas, both with a highly polylobated nucleus. In 'flower cells', the nucleus resembles the petals of a flower, while 'Atly' cells have lobes that are not arranged in any particular shape. Some interesting points to comment on include, the presence of these cells in Rottnest Island quokkas but in

quokkas on the mainland, and the presence of these cells in humans infected with HTLV-1 a deltaretrovirus. The reasons for the presence of flower cells in Rottnest Island animals but not in mainland animals remain unknown. Two options are possible, that animals on Rottnest Island are being immunostimulated to a greater degree than animals on the mainland, perhaps due to a combination of heavier loads of microfilariae, the presence of chronic infections with serovars of the *Salmonella enterica* subsp. *enterica* group, restricted diets, and human interactions that mainland animals appear to not have. Indeed the morphology of flower cells differ from that of typical reactive LYMPH stained with Wright's Giemsa (described in the quokka and other marsupial species Clark 2004), however, there is no evidence to support that these cells are nothing more than reactive LYMPH under stimulation. Alternative, flower cells are neoplastic cells undergoing nuclear changes due to the effects of a retrovirus, as cells with evident morphological similarity have been observed in humans infected with HTLV-1 a deltaretrovirus, and are considered by some authors as being a sign of a pre-leukaemic or leukaemic state (Chang *et al.* 2008 ; de Oliveira *et al.* 2010 ; Shimoyama *et al.* 1983). If this were to be the case, then quokkas and the population of Rottnest Island could become the first case of a widespread retroviral infection in macropods, with the likely possibility of being a immunosuppressive condition such as that in koalas (*Phascolarctos cinereus*), which in theory would make infected animals more prone to infection and severe disease. However, once more, there is no evidence to support that flower cells are neoplastic in nature and associated to a retroviral infection.

Nevertheless, from a conservation medicine point of view and wildlife population management, the questions surrounding flower cells in quokkas on Rottnest Island must be answered, and thorough studies should be carried out to conclusively establish what flower cells are and signify in the quokka.

8.3.4.4 Vitamin E

The low plasma concentrations of vitamin E for Rottnest Island animals are likely to represent a state of vitamin E deficiency, evident in the more prevalent oxidative damage to red blood cells for Rottnest Island animals than animals on the mainland. Some of the possible implications of oxidative injury in animals on Rottnest Island have been previously outlined in section 8.3.4.2

Low circulating vitamin E is a significant problem for captive animals, with values in Rottnest Island animals now in captivity being at least 50.4% below the lower limit (2.0-

3.2 mg/L) (Vogelnest & Portas 2010), and at least 69.5% below the upper limit, of the reference interval calculated herein for wild animals on Rottnest Island (reference interval 4.03-10.5 mg/L). The values for captive animals reported by Vogelnest and Portas (2010) are at least 162% lower for the lower limit of the reference interval calculated for wild mainland animals, and at least 416% lower for the upper limit (5.23-16.5 mg/L). Vitamin E for captive macropods is given in the form of a supplement. The low levels indicate that supplementation of vitamin E for quokkas in captivity is insufficient, with animals in captivity consequently being at greater risk of tissue damage due to oxidative injury. This may explain the high incidence of fat necrosis in captive quokkas. In conclusion, it appears necessary to recommend a readjustment of the amount of vitamin E given to quokkas in captive conditions, to achieve circulating plasma concentrations of ~9.92 mg/L.

8.4 Interrelations of concomitant infections with HMT and BLC for Rottnest Island and mainland *S. brachyurus*

The following analyses were carried out in an attempt to determine and visualise the effect if any, of concomitant infections on the HMT and BLC profiles of Rottnest Island and mainland quokkas. The intended goal is not to explain the mechanisms responsible for such differences, but simply to visualise possible patterns. Henceforth, the reader is reminded that the results obtained through these analyses are not proof of causality.

8.4.1 *Methods*

To obtain a better picture of possible patterns between concomitant infections and HMT and BLC profiles, HMT and BLC datasets were handled separately. The final sample used in the following analyses (for each dataset), was that of animals with complete HMT and BLC, and that were tested for the following infectious agents (IA) [i.e. *Salmonella* spp., *Cryptococcus* spp., saprophyte fungi, MaHV-6, microfilariae (circulating form of the filarial nematode:helminth *Breinlia* sp.), *Babesia* sp., *Theileria* sp., and trypanosomes]. Data on nematode eggs and *Eimeria* sp. were excluded as their inclusion would have reduced the sample size for each dataset (i.e. HMT and BLC) to less than 30 observations. Incomplete HMT or incomplete BLC, meant that some animals that were tested for all IA, were not included in the final dataset for analysis. Thus, the final sample sizes were 56 and 46 observations for HMT and BLC, respectively. These were used in the following analyses, non-metric multidimensional scaling (nMDS), Spearman Rank Order correlation matrix, and multiple regression (details on these tests are explained below). To obtain

prevalences of concomitant infections, animals positive to more than one IA that appeared in both HMT and BLC datasets were counted once, thus the final sample size included 58 animals. Haematology and BLC variables were fitted to an approximate normal distribution by transforming the data using BoxCox transform (STATISTICA v. 9.1, StatSoft Inc.) and observed values were then range-standardised to a scale between 0 and 1.

First, a non-metric multidimensional scaling (nMDS) of the HMT and BLC data was carried out, using a three-dimensional (three axes) model with Bray-Curtis similarity measure (Bray & Curtis 1957) in PAST v. 3.02 (Hammer, Harper, & Ryan 2001). Using the distances or similarities between all observations (i.e. HMT and BLC), nMDS obtains a set of coordinates (three in this case) and attempts to place all data points in the multidimensional model. These coordinates were extracted and used to carry out a nonparametric Spearman Rank Order correlation matrix (STATISTICA v. 9.1, StatSoft Inc.) to determine the correlations present between each of the axes of the nMDS model with all HMT and BLC variables. Positive correlations indicated that animals towards that side of the axis (i.e. positive) were more likely to have higher values than animals on the opposite side of the same axis. By contrast, a negative correlation indicated that animals towards that side of the axis (i.e. negative) were more likely to have lower values than animals on the opposite side of the same axis. Subsequently, multiple regression (STATISTICA v. 9.1, StatSoft Inc.) was used to determine the correlations (*R* and *F* statistics) and their strength (*Beta coefficient*), between each of the axes of the nMDS model with all IA. Due to insufficient observations (either only one or no positive observation captured in the final dataset) which would have effectively removed the likelihood of significance and correlation in any analyses, *Babesia* sp., and trypanosomes were excluded from both HMT and BLC datasets, while *Cryptococcus* spp. was excluded from the BLC dataset. *Cryptococcus* spp. and saprophytes were assessed separately in the haematology dataset, as more than one positive in each group was available for analysis. Site was included as an independent factor given differences between the HMT and BLC of Rottnest Island and mainland animals observed in previous analyses (see results section in Chapter 4, 5, 6, 7). A positive correlation signified that positive animals were likely to be positioned towards the positive side of the axis, while a negative correlation meant that positive animals were more likely to be positioned towards the negative side of the axis. Lastly, using the 3D nMDS axes coordinates for HMT and BLC datasets of positive and negative animals, 3D scatterplots were constructed for all IA. The positions of all observations in the 3D scatterplots do not change across infectious agents, as the source of the data is the same, i.e. HMT and BLC response variables. By contrast, positives and negatives do change. Animals having concomitant infections were identified by coloured polygons and circles.

To identify whether structures were present within each of the datasets (i.e. HMT and BLC with all IA), two separate unsupervised hierarchical cluster analyses using an unweighted pair-group average algorithm and an Euclidean similarity index were run (Sokal & Michener 1958). Each cluster analysis is presented in the form of a dendrogram with bootstrapping analysis with 100 replications. Dendrograms were then coupled with matrix plots of the HMT, BLC and nMDS axes coordinates data respectively. Cluster analyses and matrix plots were carried out in PAST v. 3.02 (Hammer, Harper, & Ryan 2001). The response variables used in these analyses were white blood cell counts (WBC), neutrophils (NEUT), eosinophils (EOS), basophils (BASO), lymphocytes (LYMPH), monocytes (MONO), red blood cell counts (RBC), haemoglobin (HGB), packed cell volume (PCV), corpuscular haemoglobin concentration mean (CHCM), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatine kinase (CK), gamma-glutamyl transferase (GGT), total plasma protein (PROT), albumin (ALB), globulin (GLOB), calcium (CALC), phosphorus (PHOS), cholesterol (CHOL), total bilirubin (BILT), glucose (GLUC), creatinine (CREAT), urea (UREA) and vitamin E (VIT. E). 95% confidence intervals (CI) for estimates of proportions (i.e. prevalence), were calculated using the Jeffreys model (n≥ 40) (Brown, Cai, & DasGupta 2001).

8.4.2 *Results*

Concomitant infections were present in 24 animals of 58 (41.4%, 95% CI 29.4-54.2). Overall, *Salmonella* spp., MaHV-6, *Theileria* sp. and microfilariae were the most common IA involved in concomitant infections (Table 8-1) Of the concomitant infections detected, the most prevalent were MaHV-6 + *Theileria* sp. (ten animals), and *Salmonella* spp. + microfilariae (eight animals). There was an equal proportion (i.e. 11) of animals infected with two and three IA, while only one animal (mainland) was positive to four IA (Table 8-1).

Table 8-1 Summary of concomitant infections captured in both haematology and blood chemistry datasets combined, for Rottnest Island and mainland animals. CI= confidence interval

Site	*Salmonella* spp.	Microfilariae	MaHV-6	*Theileria* sp.	*Cryptococcus* spp.	Saprophyte fungi	*Babesia* sp.	Trypanosomes	No. of animals	% of total	95% CI †
Rottnest Island	+						+		1	4.3	0.8-21.0
n= 45	+				+				1	4.3	0.8-21.0
				+	+				1	4.3	0.8-21.0
	+		+			+			2	3.60	0.7-11.0
	+	+			+				2	3.60	0.7-11.0
	+	+		+					2	3.60	0.7-11.0
	+	+							4	7.10	2.5-16.1
Mainland		+	+	+		+			1	4.3	0.8-21.0
n= 13		+	+	+					2	3.60	0.7-11.0
			+	+				+	1	4.3	0.8-21.0
			+	+					4	5.40	1.5-13.6
			+	+		+			2	3.60	0.7-11.0
			+			+			1	4.3	0.8-21.0
No. of animals with concomitant infection on Rottnest Island									*13*	22.4	13.2-34.3
No. of animals with concomitant infection on the mainland									*11*	19	10.5-30.4
Total no. of animals with concomitant infection									*24*	41.4	29.4-54.2
Total no. of animals tested									*58*		

† 95% confidence intervals calculated using Woolf's method (1955)

Spearman Rank Order correlation matrix analysis indicated a positive correlation of axis 1 with WBC, NEUT, MONO, RBC, HGB, PCV, CHCM, ALT, AST, CK, PROT, ALB, GLOB, CALC, CREAT, UREA and VIT. E. Of these, the strongest correlations were that of RBC, HGB, PROT and ALB. Axis 2 was positively correlated with PHOS and CHOL, and had a negative correlation with MONO, while axis 3 was negatively correlated with BASO, and positive correlated with GLUC and UREA. All correlations were significant at $p < 0.05$ (Table 8-2).

Table 8-2 Spearman rank order correlation results for correlations between axis 1, axis 2 and axis 3 of the nMDS 3D models for HMT and BLC response variables of Rottnest Island and mainland animals (combined sample). HMT= haematology, BLC= blood chemistry.

Dataset	3D nMDS	WBC	NEUT	EOS	BASO	LYMPH	
HMT	Axis 1	**0.646**	**0.469**	-0.198	0.253	0.249	
	Axis 2	0.206	0.177	0.061	0.121	0.067	
	Axis 3	-0.032	0.006	0.195	**-0.289**	0.064	
		MONO	RBC	HGB	PCV	CHCM	
	Axis 1	**0.403**	**0.838**	**0.831**	**0.670**	**0.341**	
	Axis 2	**-0.320**	0.108	0.071	-0.031	0.104	
	Axis 3	-0.240	-0.150	-0.191	-0.071	-0.262	
BLC		ALP	ALT	AST	CK	GGT	
	Axis 1	-0.165	**0.516**	**0.482**	**0.556**	**0.043**	
	Axis 2	-0.010	**-0.341**	**-0.328**	-0.245	-0.239	
	Axis 3	0.269	0.155	0.216	0.219	0.045	
		PROT	ALB	GLOB	CALC	PHOS	
	Axis 1	**0.775**	**0.780**	**0.598**	**0.408**	0.146	
	Axis 2	-0.128	0.033	-0.162	-0.244	**0.434**	
	Axis 3	0.124	0.107	0.107	-0.112	0.010	
		BILT	GLUC	CHOL	CREAT	UREA	VIT. E
	Axis 1	0.191	0.203	0.272	**0.383**	**0.332**	**0.610**
	Axis 2	-0.011	-0.153	**0.320**	-0.124	-0.124	-0.075
	Axis 3	-0.243	**0.339**	-0.262	-0.081	**0.352**	-0.186

Significant correlations ($p < 0.05$) between 3D nMDS axes and infectious agents were obtained in the multiple regression analyses, for *Salmonella* spp., saprophyte fungi, MaHV-6, *Theileria* sp., and microfilariae, but not for *Cryptococcus* spp. (Table 8-3). Similarly, there were significant correlations of axes 1 and 3 with site in the HMT response variables (Table 8-3a), and of axes 1, 2, and 3 with site in the BLC response variables (Table 8-3b).

Table 8-3 Multiple regression results for correlations between axes 1, 2, and 3 of the 3D nMDS models for HMT and BLC response variables, with site and selected infectious agents. HMT= haematology, BLC= blood chemistry.

	Dataset	3D nMDS	Infectious agent	Beta	*p*
a.	HMT	Axis 1	Site	**0.293**	**0.015**
	n= 56	R= 0.912	*Salmonella* spp.	**-0.327**	**0.001**
		F (7,48)= 33.9	*Cryptococcus* spp.	0.050	0.428
			Saprophyte fungi	0.148	0.064
			MaHV-6	0.221	0.077
			Theileria sp.	**0.274**	**0.002**
			Microfilariae	**0.136**	**0.033**
		Axis 2	Site	-0.160	0.139
		R= 0.926	*Salmonella* spp.	**-0.136**	**0.035**
		F (7,48)= 41.4	*Cryptococcus* spp.	-0.057	0.320
			Saprophyte fungi	**0.220**	**0.004**
			MaHV-6	0.188	0.101
			Theileria sp.	**-0.233**	**0.003**
			Microfilariae	**-0.768**	**0.001**
		Axis 3	Site	**-0.364**	**0.002**
		R= 0.916	*Salmonella* spp.	**-0.780**	**0.001**
		F= (7,48)= 35.8	*Cryptococcus* spp.	-0.028	0.642
			Saprophyte fungi	**-0.207**	**0.010**
			MaHV-6	**-0.262**	**0.034**
			Theileria sp.	**0.231**	**0.006**
			Microfilariae	**-0.176**	**0.006**
b.	BLC	Axis 1	Site	**0.521**	**0.001**
	n= 46	R= 0.911	*Salmonella* spp.	-0.030	0.683
		F (6,39)= 31.7	Saprophyte fungi	**0.250**	**0.011**
			MaHV-6	0.029	0.824
			Theileria sp.	**0.322**	**0.001**
			Microfilariae	**0.207**	**0.005**
		Axis 2	Site	**-0.317**	**0.006**
		R= 0.923	*Salmonella* spp.	**-0.740**	**0.000**
		F (6,39)= 37.3	Saprophyte fungi	-0.150	0.092
			MaHV-6	-0.128	0.290
			Theileria sp.	**0.460**	**0.001**
			Microfilariae	**-0.231**	**0.001**
		Axis 3	Site	-0.081	0.613
		R= 0.824	*Salmonella* spp.	-0.093	0.360
		F (6,39)= 13.7	Saprophyte fungi	**0.279**	**0.036**
			MaHV-6	0.248	0.164
			Theileria sp.	-0.143	0.241
			Microfilariae	**-0.619**	**0.001**

There was an obvious clustering of the HMT and BLC data by function of site (clusters A and B, Figures 8-1 and 8-2), showing that mainland animals generally presented higher values in both HMT and BLC analytes, than animals on Rottnest Island (heat maps, Figures 8-1 and 8-2).

Two clusters appeared to be evident in the HMT dendrogram for Rottnest Island animals, grouping the animals by those infected with *Salmonella* spp. and microfilariae (cluster C, Figure 8-1) and those that were not (cluster D, Figure 8-1). Generally, animals positive to *Salmonella* spp. and microfilariae appeared to have lower values across all HMT analytes

(heat map, Figure 8-1). This was also observed in the 3D scatterplots, where animals positive to microfilariae and *Salmonella* spp., appeared to have lower values for WBC, NEUT, MONO, RBC, PCV and CHCM, when compared to microfilariae-positive animals that were either positive to other IA different than *Salmonella* spp., or just positive to microfilariae (Figure 8-3). Visual exploration of the next most common organisms involved in single infections and coinfection (i.e. MaHV-6 and *Theileria* sp.), it can be seen that when *Salmonella* spp. and microfilariae were present either as a coinfection or as a single infection, the HMT profiles of animals with MaHV-6 and *Theileria* sp. either as a single infection or as coinfections, were different than those of MaHV-6-*Theileria* sp. coinfected with another IA (Figure 8-4). This was the case for both Rottnest Island in which *Salmonella* spp. and microfilariae were detected, and mainland animals in which microfilariae was detected but not *Salmonella* spp. There were no other apparent differences by function of other organisms.

Similarly to the Rottnest Island case, cluster analysis grouped mainland animals in two clusters, those that were positive to saprophyte fungi and other IA (mostly MaHV-6 and *Theileria* sp., cluster E: Figure 8-1) and those that were not positive to saprophyte fungi but positive to other IA (mostly MaHV-6 and *Theileria* sp., cluster F: Figure 8-1). However, no apparent patterns in the HMT of these animals were observed in heat maps (Figure 8-1) or 3D scatterplots (Figures 8-3, 8-4, and 8-5 right) by function of saprophyte fungi.

There were no evident clusters in the BLC analysis of Rottnest Island animals (Figure 8-2). However, 3D scatterplots did show that animals positive to *Salmonella* spp. and microfilariae appeared to have a different (lower) BLC profiles than animals that were only positive to *Salmonella* spp., or positive to Salmonella spp. and other organisms (Figure 8-7 left). By contrast, Rottnest Island animals with microfilariae single infection appeared to have different (lower) BLC profiles than Rottnest Island animals with *Salmonella* spp. single infection. This was the case for all animals infected with microfilariae, either as a single infection or as coinfection (Figure 8-7 right). By contrast, cluster analysis grouped mainland animals by those positive to MaHV-6, *Theileria* sp. and microfilariae (cluster C, Figure 8-2) and those that were positive to MaHV-6 and *Theileria* sp. but not to microfilariae (cluster D, Figure 8-2). A subtle difference between the BLC of animals in these two clusters was observed in the 3D scatterplots (Figure 8-8), whereby animals in cluster C appeared to have a different (lower) BLC profile than animals in cluster D. There were no other apparent BLC patterns in animals with other concomitant infections.

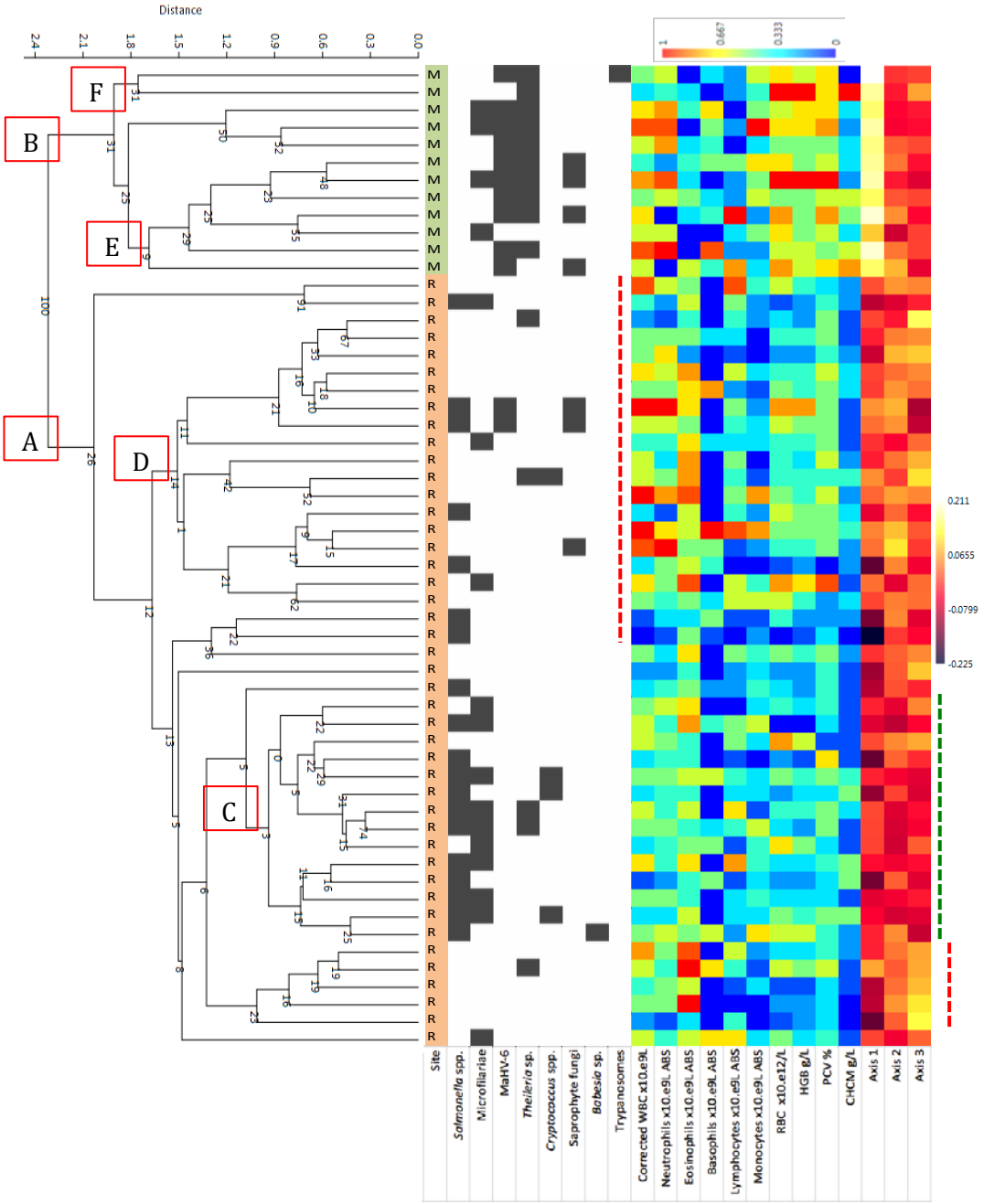


Figure 8-1 Cluster analysis and patterns of 10 HMT analytes of 44 animals from Rottnest Island (R) and 12 animals from the mainland (M) that were tested for eight infectious agents (*Salmonella* spp., microfilariae, MaHV-6, *Theileria* sp., *Cryptococcus* spp., saprophyte fungi, *Babesia* sp., and trypanosomes). Clusters A (Rottnest Island), B (mainland), C (coinfection with *Salmonella* spp. and microfilariae), D (no coinfection with *Salmonella* spp. and microfilariae), E (coinfection with saprophyte fungi and other organisms) and F (coinfection with other organisms but not with saprophyte fungi). General patterns in axes 1, 2 and 3 combined showed differences in *Salmonella* spp.-microfilariae negative animals (red doted lines), and *Salmonella* spp.-microfilariae positive animals (green doted line).

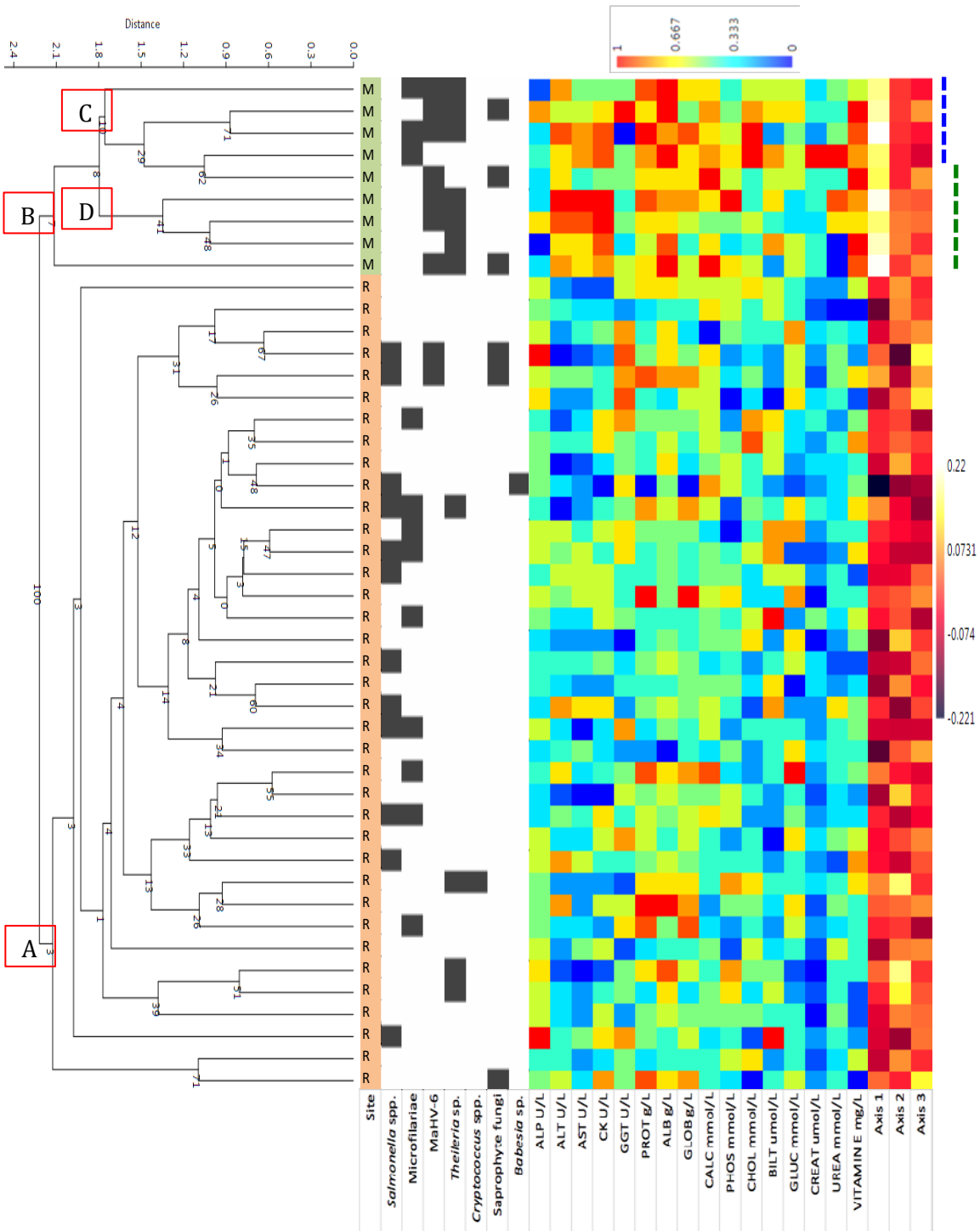


Figure 8-2 Cluster analysis and patterns of 16 BLC analytes of 47 animals from Rottnest Island (R) and 9 animals from the mainland (M) that were tested for seven infectious agents (*Salmonella* spp., microfilariae, MaHV-6, *Theileria* sp., *Cryptococcus* spp., saprophyte fungi and *Babesia* sp. Trypanosomes were not included as there were no positive animals). Clusters A (Rottnest Island), B (mainland), C (microfilariae-negative and coinfection with MaHV-6, *Theileria* sp. and saprophyte fungi), and D (microfilariae-positive and coinfection with MaHV-6, *Theileria* sp., and saprophyte fungi). General patterns in axes 1, 2, and 3 combined showed differences in microfilariae-positive animals (blue doted line), and microfilariae-negative animals (green doted line.

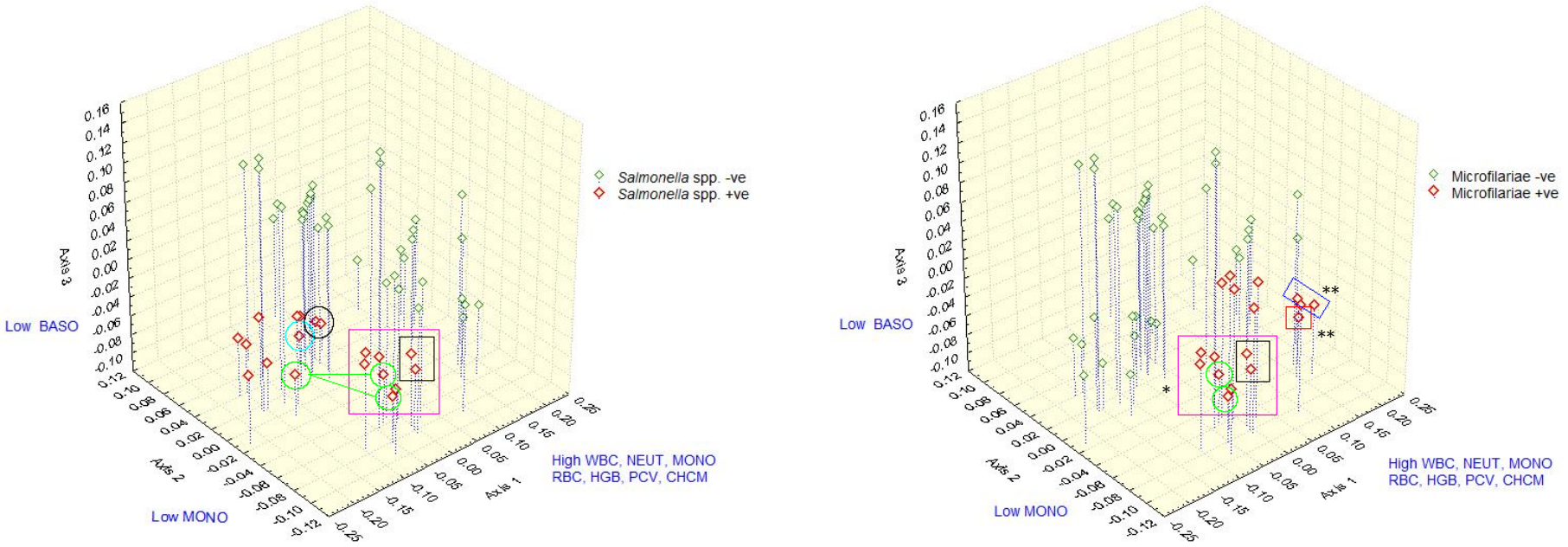


Figure 8-3 3D scatterplots of axes 1, 2, and 3 coordinates of the nMDS analysis for HMT profiles of animals positive to *Salmonella* spp. (left), and animals positive to microfilariae (right). Animals with concomitant infections are indicated by polygons and circles. Polygons: pink= *Salmonella* spp. and microfilariae, black= *Theileria* sp., red= MaHV-6, *Theileria* sp., and saprophyte fungi, dark blue= MaHV-6 and *Theileria* sp.; Circles: green= *Cryptococcus* spp., black= MaHV-6 and saprophyte fungi, light blue= *Babesia* sp. *= Rottnest Island, **= mainland. Animals only positive to *Salmonella* spp. are from Rottnest Island. Animals only positive to microfilariae are from both Rottnest Island (4) and mainland (1).

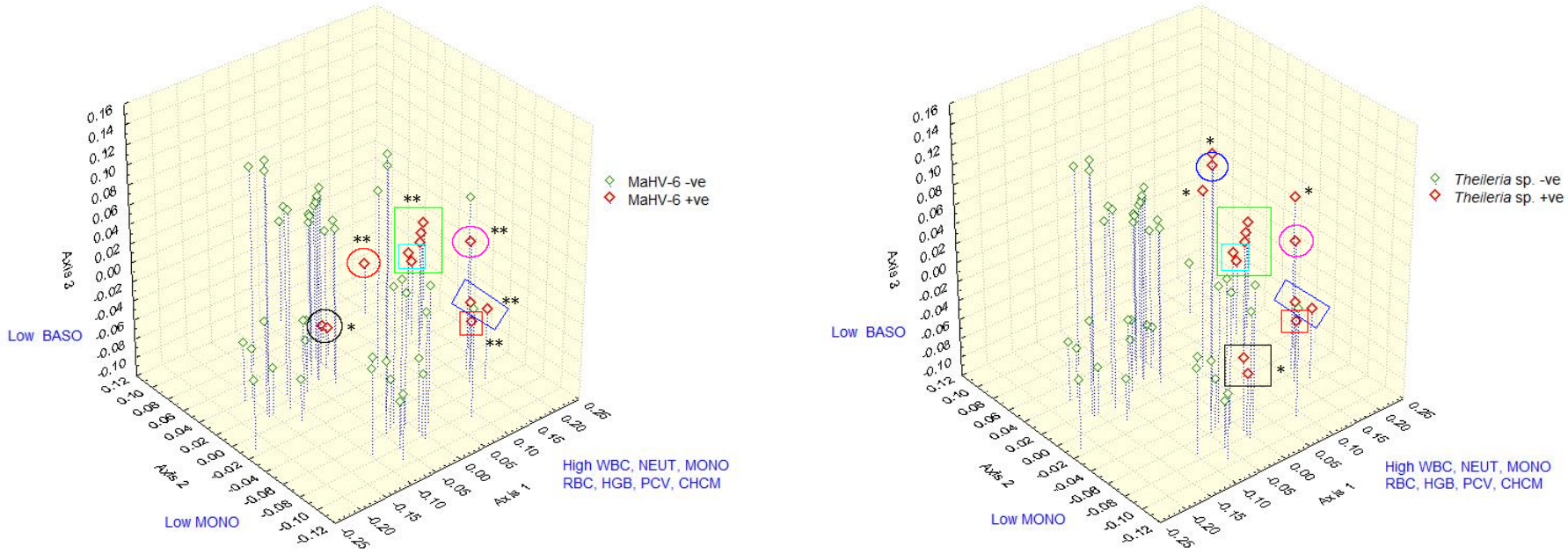


Figure 8-4 3D scatterplots of axes 1, 2, and 3 coordinates of the nMDS analysis for HMT profiles of animals positive to MaHV-6 spp. (left), and animals positive to *Theileria* sp. (right). Animals with concomitant infections are indicated by polygons and circles. Polygons: green= MaHV-6 and *Theileria* sp., red= microfilariae and saprophyte fungi, dark blue= microfilariae, black= *Salmonella* spp., and microfilariae, light blue= saprophyte fungi; Circles: black= *Salmonella* spp. and saprophyte fungi, red= saprophyte fungi, pink= trypanosomes, dark blue= *Cryptococcus* spp. *= Rottnest Island, **= mainland.

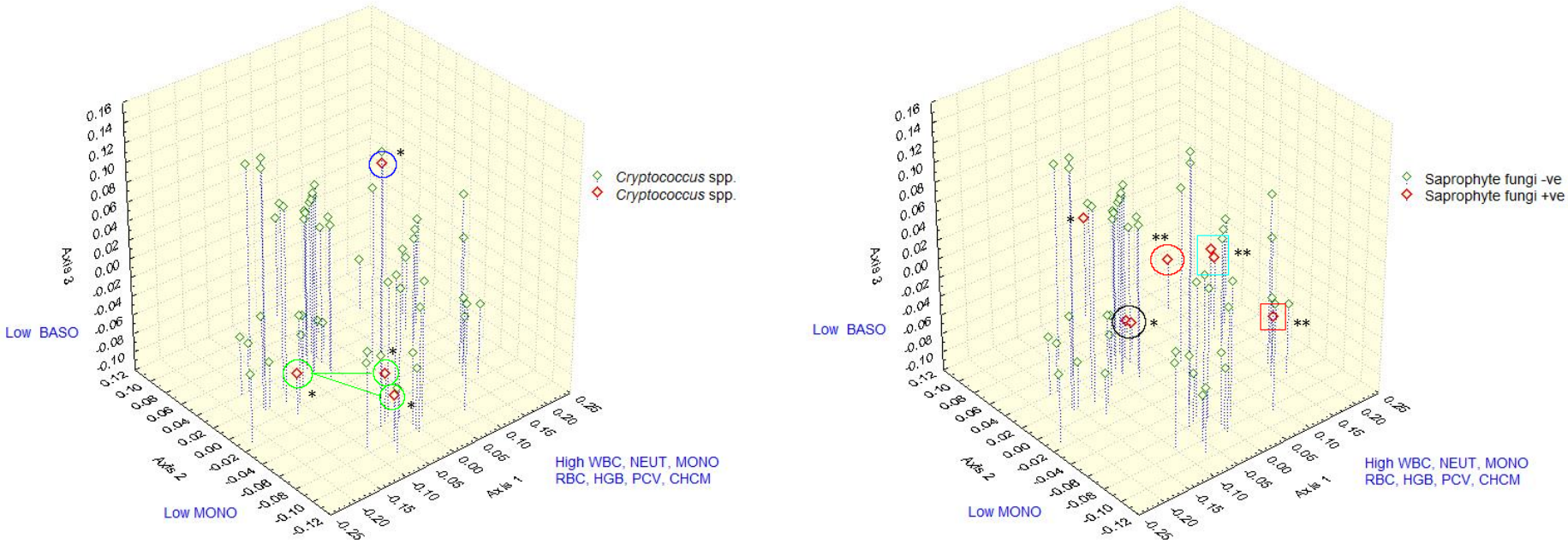


Figure 8-5 3D scatterplots of axes 1, 2, and 3 coordinates of the nMDS analysis for HMT profiles of animals positive to *Cryptococcus* spp. (left), and animals positive to saprophyte fungi (right). Animals with concomitant infections are indicated by polygons and circles. Polygons: light blue= MaHV-6 and *Theileria* sp., red= MaHV-6, *Theileria* sp., and microfilariae; Circles: green= *Cryptococcus* spp., *Salmonella* spp., and microfilariae, dark blue= *Theileria* sp., black= MaHV-6 and *Salmonella* spp., red= MaHV-6. *= Rottnest Island, **= mainland.

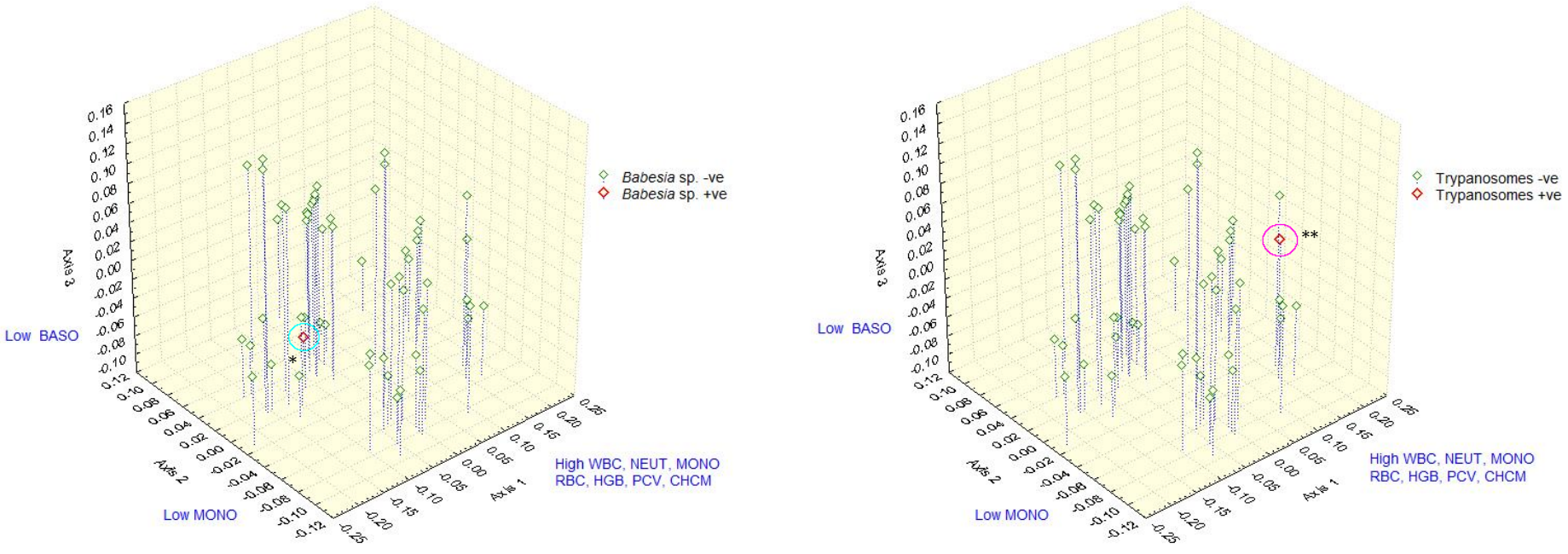


Figure 8-6 3D scatterplots of axes 1, 2, and 3 coordinates of the nMDS analysis for HMT profiles of animals positive to *Babesia* sp. (left), and animals positive to trypanosomes (right). Animals with concomitant infections are indicated by circles; light blue: *Salmonella* spp., pink= MaHV-6 and *Theileria* sp. *= Rottnest Island, **= mainland.

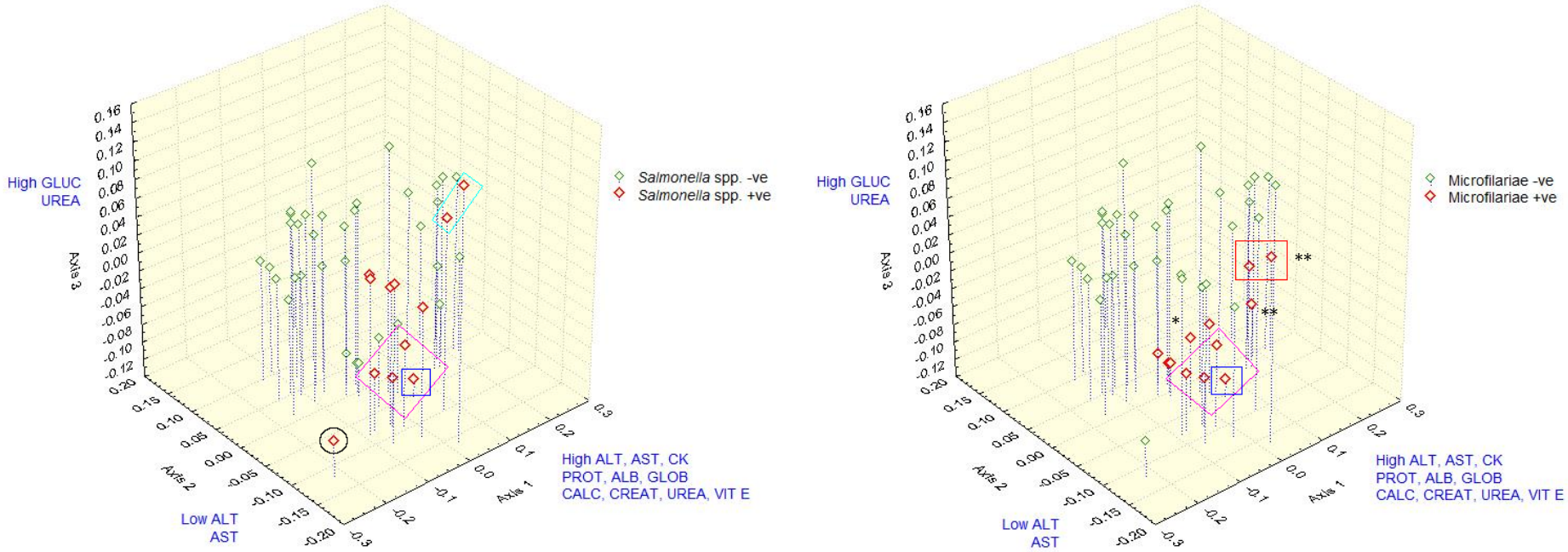


Figure 8-7 3D scatterplots of axes 1, 2, and 3 coordinates of the nMDS analysis for BLC profiles of animals positive to *Salmonella* spp. (left), and animals positive to microfilariae (right). Animals with concomitant infections are indicated by polygons and circles. Polygons: pink= *Salmonella* spp. and microfilariae, dark blue= *Theileria* sp., light blue= MaHV-6 and saprophyte fungi, red= MaHV-6 and *Theileria* sp.; Circles: black= *Babesia* sp. All animals positive to *Salmonella* spp. are from Rottnest Island. *= Rottnest Island, **= mainland.

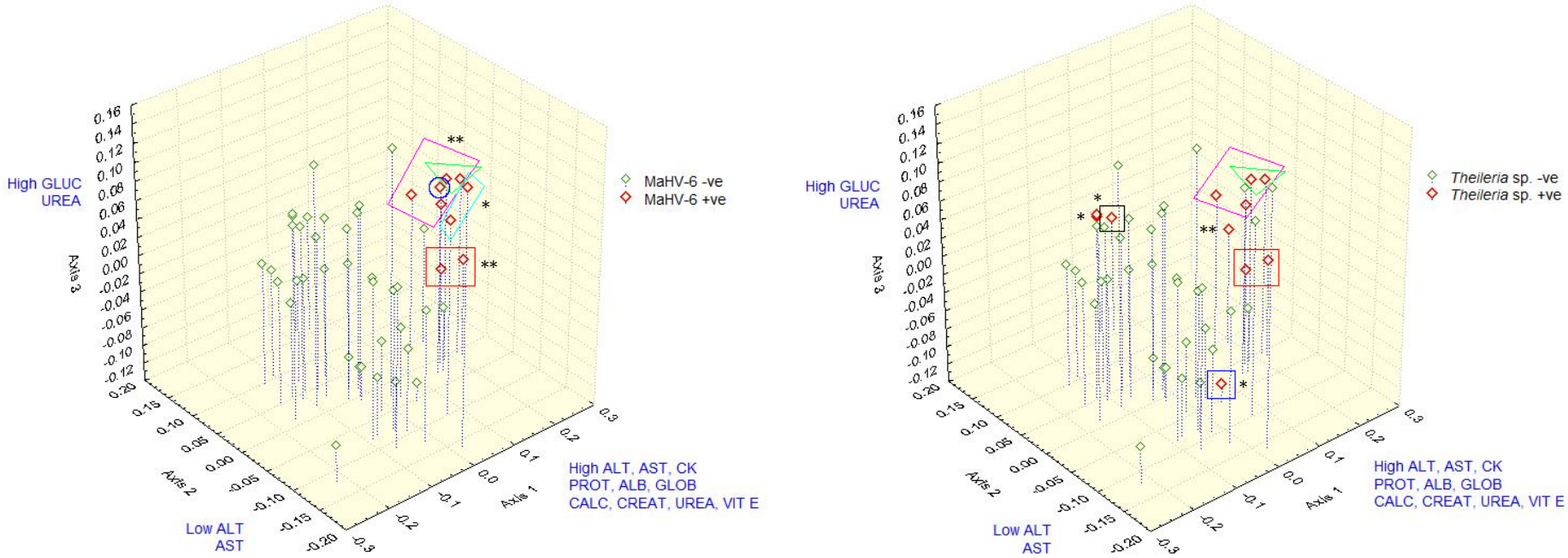


Figure 8-8 3D scatterplots of axes 1, 2, and 3 coordinates of the nMDS analysis for BLC profiles of animals positive to MaHV-6 (left), and animals positive to *Theileria* sp. (right). Animals with concomitant infections are indicated by polygons and circles. Polygons: pink= MaHV-6 and *Theileria* sp., green= saprophyte fungi, light blue= *Salmonella* spp. and saprophyte fungi, red= microfilariae, dark blue= *Salmonella* spp. and microfilariae, black= *Cryptococcus* spp.; Circles: dark blue= saprophyte fungi. *= Rottnest Island, **= mainland.

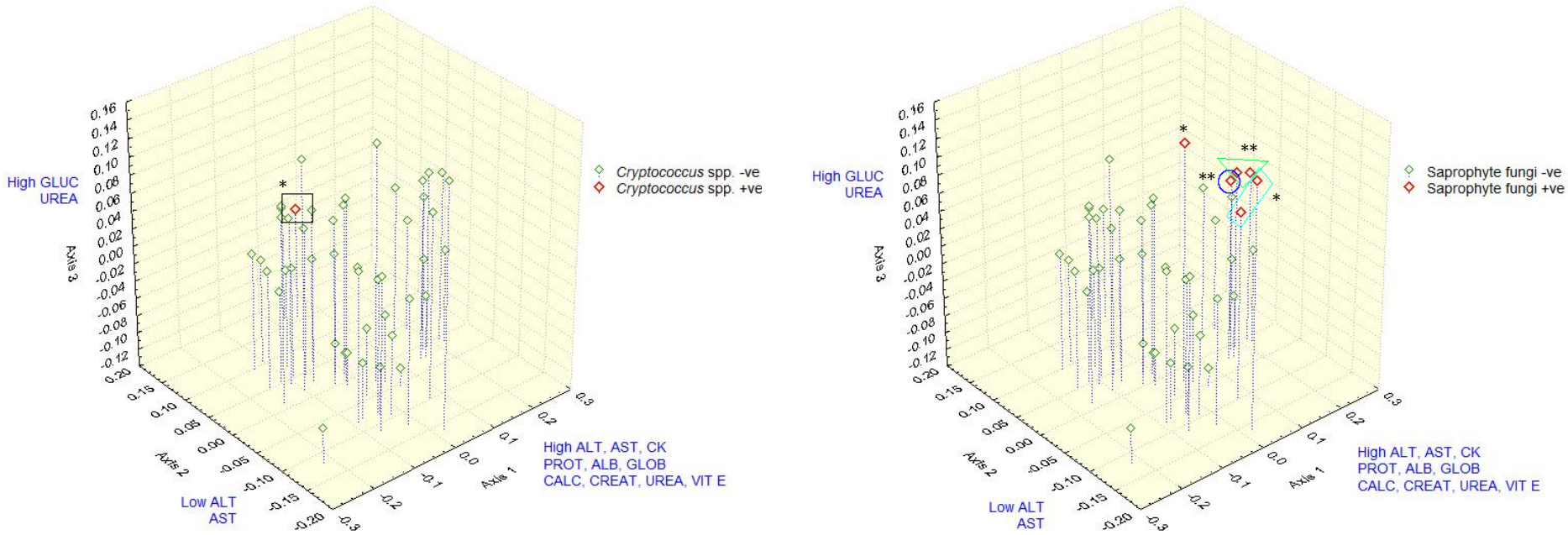


Figure 8-9 3D scatterplots of axes 1, 2, and 3 coordinates of the nMDS analysis for BLC profiles of animals positive to *Cryptococcus* spp. (left), and animals positive to saprophyte fungi (right). Animals with concomitant infections are indicated by polygons and circles. Polygons: black= *Theileria* sp., green= MaHV-6 and *Theileria* sp., light blue= *Salmonella* spp. and MaHV-6; Circles: dark blue= MaHV-6. *= Rottnest Island, **= mainland.

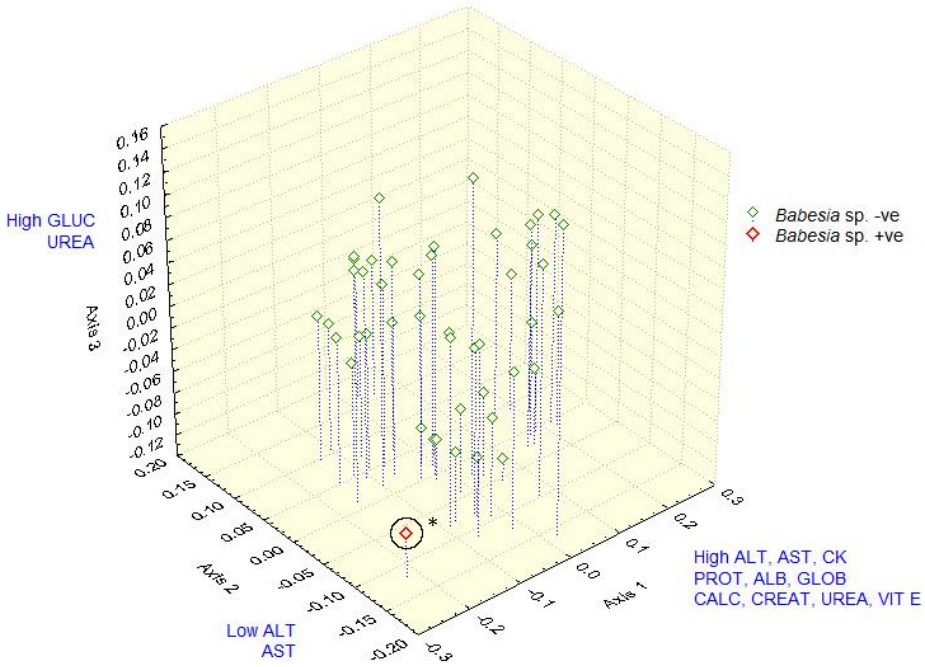


Figure 8-10 3D scatterplots of axes 1, 2, and 3 coordinates of the nMDS analysis for BLC profiles of animals positive to *Babesia* sp. Animals with concomitant infections are indicated by circles. Circles: black= *Salmonella* spp. *= Rottnest Island.

8.4.3 *Discussion*

The distinctive clusters by site (A and B) in both the HMT and BLC cluster analyses indicating mainland and Rottnest Island animals have different HMT and BLC parameters, is in line with previous analyses carried out in this project (see Chapter 7 section 7.4.1), and with previous studies in other Australian wildlife species (Clark *et al.* 2003 ; Clarke *et al.* 2013 ; King & Bradshaw 2010 ; Pacioni *et al.* 2013). These differences may be attributed to the interplay of multiple factors (e.g. diet, climate, predator presence, underlying disease) that vary between these two populations, as it has been proposed by other authors (Bennett *et al.* 2007 ; Clark 2004 ; Clark *et al.* 2003 ; Clarke *et al.* 2013 ; King & Bradshaw 2010 ; McKenzie, Deane, & Burnett 2002 ; Pacioni *et al.* 2013 ; Robert & Schwanz 2013).

Of all coinfections present in quokkas, interactions between IA and effects on the host appeared to be more evident for coinfections with *Salmonella* spp.-microfilariae (Rottnest Island), and MaHV-6-*Theileria* sp. (mostly in mainland animals). Other coinfections may have not been clustered in the analyses, not due to the absence of interactions between the IA involved in such coinfections, or the absence of patterns in their corresponding HMT and BLC profiles, but most likely as a result of a small sample size (i.e. insufficient number of cases). This would be the case of coinfections such as *Salmonella* spp.-microfilaria-*Cryptococcus* spp. (three animals), *Salmonella* spp.-MaHV-6-saprophyte fungi (two animals), *Theileria* sp.-*Cryptococcus* spp. (one animal), and MaHV-6-*Theileria* sp.-trypanosomes (one animal).

Clusters for the HMT of the Rottnest Island sample (Figure 8-1), grouped the animals by those that presented coinfection with *Salmonella* spp. and microfilaria (cluster C), and those that were positive either to *Salmonella* spp. or microfilariae, or other IA different than *Salmonella* spp. (cluster D); this is suggestive of an interaction between these two infectious agents, and its in line with results previously obtained for *Salmonella* spp. and microfilariae in this study, in which quokkas were more likely to be positive to *Salmonella* spp. if coinfected with microfilariae (OR= 3.88, 95% CI 1.31-11.5, p= 0.012) (see Chapter 4, section 4.3.2). Interestingly, results of the 3D scatterplots and correlation analysis for the HMT component, appeared to suggest that *Salmonella* spp. may have a greater involvement in the low HMT seen in *Salmonella* spp.-microfilariae positive animals, than microfilariae (Figure 8-3), given that animals positive to other IA but not to *Salmonella* spp. presented HMT profiles with higher values. The impact of this interaction (*Salmonella*

spp.-microfilariae) on the host, is further seen in the BLC profiles of infected animals (Rottnest Island) (Figure 8-7), where animals coinfected with *Salmonella* spp. and microfilariae showed different BLC profiles (for the analytes included by the correlation analysis) when compared to other coinfections. However, in this case it appears that microfilariae may have a greater involvement in the different BLC profiles seen in *Salmonella* spp.-microfilariae positive animals, as animals that were positive only to *Salmonella* spp. appeared to have higher BLC than those that were only positive to microfilariae. However, considering the methods used to detect *Salmonella* spp. (culture) and microfilariae (light microscopy), false negative animals may have been among the obtained results. This means that it is possible that animals that were only positive to microfilariae or *Salmonella* spp. may have been positive to both. Thus, it is plausible that both organisms are involved in the HMT and BLC parameters observed. It is unknown why this interaction and its effect on BLC parameters were not highlighted by the cluster analysis. Interestingly, the effect of *Salmonella* spp. and microfilariae on HMT profiles can be further observed even in the presence of other coinfections. This is the case of the HMT profiles of animals coinfected with MaHV-6 and *Theileria* sp. (both Rottnest Island and mainland), that appear to be different when *Salmonella* spp. and/or microfilariae are present. In both, that is wether *Salmonella* spp. is involved, or whether microfilariae is involved, HMT analytes appeared to be different (lower).

Clusters E, F (HMT: Figure 8-1) and clusters C and D (BLC: Figure 8-2) obtained for the mainland sample, are characterised by a greater number of coinfections with MaHV-6 and *Theileria* sp. A significant association (p= 0.001) between MaHV-6 and *Theileria* sp. has already been obtained in this project, in which MaHV-6 positive animals were 11 times more likely to be infected with *Theileria* sp. (OR= 11.0, 95% CI 2.31-52.3) than MaHV-6 negative ones (see Chapter 6, section 6.4.5). The inclusion of saprophyte fungi in cluster E but not in cluster F (HMT analysis), may be suggestive of an interaction between these IA and MaHV-6 and *Theileria* sp., and that the BLC profiles of animals coinfected with MaHV-6 and *Theileria* sp. may be sufficiently different when coinfected with saprophyte fungi. However, there were no apparent patterns in the HMT and BLC profiles by function of saprophyte fungi of these animals. Alternatively, the apparent involvement of saprophyte fungi in the clustering of the HMT of mainland animals may be an artefact of the small mainland sample size. Similarly, the inclusion of microfilaria in cluster C but not in cluster D (BCL analysis) may suggest an interaction between microfilaria and MaHV-6 and *Theileria* sp., and that the BLC profiles of animals coinfected with MaHV-6 and *Theileria* sp. may vary sufficiently when also coinfected with microfilariae. Lower BLC was observed in animals infected with MaHV-6 and *Theileria* sp. that were coinfected with microfilariae

suggesting that the presence of this helminth infection generates further impact on the BLC of these mainland animals.

Coinfections with bacteria, viruses, helminths, and protozoans have been previously reported (mostly studied in pairs), and both synergistic and antagonistic interactions have been observed with various effects on the host (Cox 2001), however, it is generally understood that immunosuppression in different degrees is a common factor to them. Coinfections with helminths (such as *Breinlia* sp. which has been previously reported in Rottnest Island animals, and for which circulating microfilariae were detected in peripheral blood of Rottnest Island and mainland quokkas by this project), appear to enhance bacterial, viral and protozoal infections (Cox 2001). In helminth and bacterial coinfections, helminths have been reported to facilitate and enhance the bacterial component. Such is the case of filarial and *M. tuberculosis* coinfection (Salgame, Yap, & Gause 2013), and nematodes and *Salmonella enterica* ser. Typhimurium coinfection (Su *et al.* 2014). Similarly, coinfection studies on gammaherpesviruses (such as MaHV-6) which are characterised by establishing latent infections, or infections that are "dormant", and helminths, have shown that the latter can induce viral reactivation if T helper cells Type 2 inflammation is present (Reese *et al.* 2014). Helminths and protozoans (e.g. *Theileria* sp. and *Babesia* sp.) have been reported to have complex interactions in the coinfected host, both synergistic (Dwinger *et al.* 1994) and antagonistic (Behnke, Sinski, & Wakelin 1999). In relation to viral and protozoal infections, studies have shown that in concomitant infection, both tend to act synergistically thus prompting worse clinical conditions, and have been associated with reduced body condition and higher mortality rates in laboratory animals (Bordes & Morand 2011), and higher mortality rates in wild mammals (Munson *et al.* 2008) and birds (Alley *et al.* 2010). Although there is no irrefutable evidence, it may be reasonable to considered that in the case of the quokka, if immunosuppression is taking place, the helminth and the viral components of their complex coinfections may be playing a greater role in such immunosuppressed state (Bordes & Morand 2011 ; Cox 2001) and may be favouring infections by other type of organisms (Behnke, Sinski, & Wakelin 1999 ; Mishra *et al.* 2014 ; Reese *et al.* 2014 ; Semnani *et al.* 2006 ; Semnani *et al.* 2008).

However, these multi-factorial multi-pathogenic systems do not work one way, but are more dynamic, probably too dynamic that understanding the mechanisms responsible for the clinical status of the host, although not impossible, is a complex task. For instance, while helminth infections can induce viral reactivation of a latent gammaherpesvirus infection (e.g. MaHV-6 in both Rottnest Island and mainland animals), that same latent

infection may be playing its role in allowing infected animals to cope with other challenges. Studies in animal models have shown that gammaherpesviral latent infection can lead to an enhanced immune state in the host, which in turn protects the host against other challenges (Reese 2016). Is this one of the possible reasons why quokkas on Rottnest Island and the mainland may be able to cope with their complex coinfections?

Some subtle discrepancies were observed between the HMT and BLC results obtained in the 3D scatterplots-correlation analyses for Rottnest Island and mainland groups (concomitant infection analyses), and those previously obtained (single infection analyses) through non-parametric multivariate analyses (e.g. PERMANOVA and ANOSIM) for *Salmonella* spp. (see section 4.3.3) and MaHV-6 (see section 6.4.4). Possible explanations may include: i) previous analyses did not take into account concomitant infection, thus some of the differences seen in the current section may be attributed to the infection with a second organism, ii) difference in sample sizes, as both HMT and BLC datasets used in the current concomitant infection analyses used less animals than in previous analyses, and iii) innate differences in the statistical properties of the various statistical techniques used.

8.4.4 *Conclusion*

Interactions between infectious agents and their effect on the host are characterised by unpredictability. It can not be any other way given the many factors involved in concomitant infections. Some of these factors are extrinsic in nature (e.g. diet, climate, changing habitat, predators), and other ones are intrinsic in nature [e.g. immune and physiological status, micro/macroparasite load, types of organisms involved (virus, bacteria, fungi, protozoa, helminth), the nature of the first infection (chronic or acute) when the second is acquired] (Cox 2001). Although all microparasites detected in this study and the possible implications of their intricate interactions should ideally be taken into account at the moment of generating an overall impression of the clinical status of quokkas on Rottnest Island and the mainland, some findings appear to be more worth noting than others, namely *Salmonella* spp., microfilariae, MaHV-6, *Theileria* sp., flower cells in peripheral blood, regenerative normocytic hypochromic anaemia, and lower plasma levels of vit. E. Of these, only microfilariae, MaHV-6, and *Theileria* sp. apply to mainland animals. "Adding bags of sand to a rowboat until it sinks" was the analogy used by Rhyan and Spraker (2010) when referring to the impact of mounting stressors on disease transmission cycles. In line with Rhyan and Spraker's analogy, there are several

bags of sand already piling up in quokkas, and these "bags of sand" are known for altering the only machinery by which quokkas could cope with current and further challenges, their immune system.

Although the following attempt lacks evidence and therefore is theoretical in essence, it is still important to reconstruct an overall picture of what may be happening in quokkas on Rottnest Island and the mainland. *Salmonella* spp. and microfilariae were found to be intimately related and appear to exert the heavier effect on quokkas on Rottnest Island, with a possible downregulating effect of both agents on the immune system of the animals (lower WBC in *Salmonella* spp. and microfilariae infected animals). However, it is likely that the helminth infection is altering the immune system of the animals to a greater degree than *Salmonella* spp. and consequently favouring the bacterial infection, as it has been previously reported in other species. While this interaction and its downregulating effect is taking place on the host, MaHV-6 and *Theileria* sp. are found to be also intimately related, with MaHV-6 positive animals having a greater likelihood of being infected with the protozoan, perhaps due to a possible immunosuppressive effect of the virus that specifically benefits the protozoan. Bridging across these coinfections in Rottnest Island and mainland animals, is microfilariae that has not only been associated with enhancing bacterial infections, but have also been associated with the reactivation of latent gammaherpesviral infections, such as MaHV-6 detected in animals from Rottnest Island and the mainland. Interestingly, even though there were no significant differences in the prevalence of microfilariae between Rottnest Island and mainland animals, the degree of microfilaraemia was higher in the Rottnest Island group when compared to the mainland. In the context of protozoans (i.e. *Theileria* sp. ad *Babesia* sp.), the observed number of positive cases on Rottnest Island were insufficient for the statistical analyses to be able to detect any particular pattern in the HMT and BLC of infected animals, while on the mainland, the overall sample was too small, and the number of negative cases where insufficient to compare patterns against positive animals. Despite this, it is likely that these protozoans are also playing their role in these coinfections and having an effect on the host.

All these infections, which could be considered to be chronic in nature, may be contributing to the anaemic state of the Rottnest Island quokka, through processes that include inflammation, and changes in iron homeostasis induced by cytokines and cells of the reticuloendothelial system (Weiss & Goodnough 2005). Furthermore, microfilariae and *Theileria* sp. may also be contributing to the anaemia, by direct damage of erythrocytes. However, filarial infection has been reported to give protection against

anaemia during malarial infection (Salgame, Yap, & Gause 2013). Anaemia may not be present on mainland animals due to better diets (e.g. higher uptake of vit. E), the absence of infection with serovars of the *Salmonella enterica* subsp. *enterica* group as seen on Rottnest Island, milder degree of microfilaraemia when compared to Rottnest Island animals, and higher levels of vit. E in plasma than their island conspecifics.

The presence of flower cells, and lower levels of vit. E in plasma of Rottnest Island animals when compared to mainland animals, although not infectious, are two more factors adding to the overall pressure that concomitant infections already represent. Flower cells and vit. E may be interconnected. To the author's knowledge, flower cells (described as lymphocyte-like cells with a flower shape nucleus) have only been detected in humans infected with human T-cell leukaemia virus type 1 (a deltaretrovirus), in both asymptomatic carriers and those with clinical disease (de Oliveira *et al.* 2010), a virus known for its immunosuppressive ability. A study by Fukuda et al. (2005) showed that the morphology of the nucleus in flower cells of patients infected with HTLV-1, is the result of microtubule constriction by *upregulation* of the phosphatidyl inositol 3-kinase, an important regulatory protein involved in control of cell growth, survival, and malignant transformation (Krasilnikov 2000). Interestingly, plasma levels of vit. E that has been linked to *downregulation* of the phosphatidylinositol 3-kinase pathway (Ni *et al.* 2005), were found to be lower in Rottnest Island animals than in mainland animals. Could downregulation of the phosphatidylinositol 3-kinase pathway be absent or impaired in Rottnest Island quokkas due to low vit. E, thus enhancing abnormal proliferation and malignant transformation? Are flower cells in peripheral blood of Rottnest Island quokkas neoplastic cells and a deltaretrovirus is involved considering the nuclear morphology of flower cells?, or are flower cells just atypical LYMPH that appear to be undergoing a greater degree of immunostimulation in response to ongoing pressures (e.g. diet shortage, human interaction, concomitant infection)? Although these questions are legitimate, there is no conclusive evidence (probably no evidence at all) against or in favour of any hypothesis (i.e. neoplastic cells that denote pathology, or atypical reactive LYMPH). For this reason, it did not appear reasonable to discuss the clinicopathological results in previous chapters in the context of an immunosuppressive disease. Furthermore, there was no significant difference in the proportion of flower cells between infected animals (i.e. *Salmonella* spp., *Cryptococcus* spp., or MaHV-6) and non infected ones. Regardless, it appears imperative to rule out a pre-leukaemic state in quokkas on Rottnest Island and further research is advisable.

Lastly, immunosuppression due to chronic exposure to increasing levels and sources of stress may be the tipping point in this concomitant infection story. The consequences of allostatic overload on the infected animals may include increased susceptibility to infection, severity of clinical disease, shedding of infectious agents (e.g. *Salmonella* spp.), probability of reactivation of latent infections (e.g. MaHV-6) or dormant lesions (such as those in *Cryptococcus neoformans* var. *grubii* infection) and poor prognosis (Hing *et al.* 2016).

8.5 Improved protocols

Disease surveillance and health monitoring are necessary tools to properly carry out conservation management of wild populations of any animal, including quokkas. These tools do not just allow the collection and maintenance of up to date epidemiological data (e.g. prevalence, incidence, and distribution of infectious agents), but also facilitate the early detection of disease outbreaks, and contribute to decision making. For this reason, the following guidelines are recommended for wild quokkas on Rottnest Island and the mainland of WA.

Animal handling. Calico bags were sufficiently sturdy to withstand hind limb claws of Rottnest Island animals, but light and manageable enough to offer great manoeuvrability of the animal from outside the bag, making it easier to restraint the animals' head, to then carry out anaesthesia induction. By contrast, calico bags are not sturdy enough to handle mainland animals and hessian bags are recommended instead.

General anaesthesia and physical examination. Anaesthesia induction of quokkas on both Rottnest Island and the mainland may be safely carried out with 5% isoflurane, delivered in 100% medical oxygen at a flow rate of 2.5L/minute. Anaesthetic depth (usually 2-3 min post induction) is accurately assessed through the loss of general muscle tone, the absence of a corneal reflex and hindlimb withdrawal response. Anaesthesia should be able to be maintained on 2-2.5% isoflurane with an oxygen flow rate of 2L/min. At 1.5% isoflurane with the same oxygen flow rate, animals regained consciousness. In addition to auscultation using a stethoscope, monitoring heart rate would be better achieved using a pulse oximeter which would also allow oxygen saturation to be monitored. Anaesthetic and general physical examination data sheets (see section 3.3) should be completed every time an individual is anaesthetised and examined. Physical examination should be undertaken every time an animal is trapped. This examination should include: eyes, ears,

nose, oral cavity, coat, body condition, body weigh, skeletal structure, external parasites, as well as inspection of the pouch, tail, testes (if male), and cloacal region.

Laboratory procedures. All laboratory protocols and procedures used in this study were effective for screening and identifying *Salmonella* spp., *C. neoformans* var. *grubii*, MaHV-6, and piroplasms (i.e. *Babesia* sp. and *Theileria* sp.). Procedures and protocols used in HMT and BLC studies were equally effective and reliable.

Blood studies. Blood can be used (section 3.4.2) for HMT, BLC, vitamin E, as well as serology for *C. neoformans (all varieties*), *C. gattii*, MaHV-6, and other infectious agents (e.g., *T. gondii*). It is recommended that, unless light scattering and impedance standards are determined for erythrocytes and white blood cells in quokkas, future automated HMT analyses could be run using the same settings used in this project (i.e. multispecies software with canine setting on an ADVIA-120® automated HMT analyser; Bayer diagnostics division, Tarrytown, New York, USA). Generally speaking, cellular morphology was still unaffected when performing blood smears within 2-3 h post collection.

Disease surveillance. Although the protocols and procedures used in this project were effective for sample collection, handling, and storage, as well as detection and identification of recovered organisms or DNA, some aspects of these protocols may be adjusted in order to improve detection and identification of the organisms in this study. For instance: i) for *Salmonella* spp. screening: faecal samples obtained through rectal palpation should be combined with cloacal wet swabs (in Amie's medium); and swabs should be cultured on brilliant green agar and bismuth sulphite agar, in addition to XLD media; blood culture should be incorporated as a standard test in Rottnest Island animals to determine whether salmonellosis is present or not, ii) for *C. neoformans* var. *grubii* screening: duplicate nasal swabs, single swabs from the ventral aspect of the animals paws, and swabs of potential environmental sources should be collected, a latex-cryptococcal agglutination antigen test (LCAT) should also be carried out, as well as PCR-finger printing to determine the molecular type of all isolates recovered.

8.6 Management Implications of this study

- Avoid long term changes to the environment which would force quokkas to be exposed to greater levels of endogenous glucocorticoids due to chronic stress, a powerful downregulator of the immune system. This applies equally to Rottnest

Island and mainland groups. Infectious organisms studied in this project (i.e. *Salmonella* spp., *C. neoformans* var. *grubii*, MaHV-6, microfilariae, *Babesia* sp. and *Theileria* sp.), are known to cause disease when the host is under chronic stress (e.g. loss of habitat, drought, food deprivation, feral animals competition).

- In addition to standard cryptococcal tests (e.g. latex cryptococcal agglutination test, culturing and PCR of nasal swabs), animals from Rottnest Island destine for captivity or other types of conservation programs (e.g. translocation) should be screened for lesions in the cribriform plate consistent with cryptococcal pathology through nasal endoscopy. However, this diagnostic technique is limited to the nasal cavity and would not answer the question of whether the animals carry pulmonary dormant lesions or not. If detecting these lesions is considered a priority, then advanced imaging techniques such as magnetic resonance imaging or computed axial tomography may be considered, as these lesions are difficult to detect with radiography, or not detected at all. This would enable identification of which animals are at a greater risk of disease, and subsequently carry out required management procedures.
- In accordance to the serious implications of *Salmonella* in public health, it is important to the Rottnest Island Authority, as well as to the Department of Health of Western Australia, to be aware of the changes in richness, abundance, prevalence and distribution of *Salmonella* serovars on the island. Although comparative studies of *Salmonella* loads in the environment and in quokkas were not carried out in this study, we support the use of quokkas as sentinel species to monitor the ecology of *Salmonella* serovars on Rottnest Island, as previous authors have proposed (Hart 1980 ; Iveson 1977).
- Given the presence of *Salmonella* serovars (especially those in the *Salmonella enterica* subsp. *enterica* group) in Rottnest Island animals that are not evident for mainland populations, movement of animals from Rottnest Island to the mainland (as has happened at least once within the City of Mandurah) should be considered a Biosecurity risk and handled accordingly.
- Agencies responsible for the conservation of Australian native fauna should consider spill-over of MaHV-6 from infected quokkas to other related species, as a possibility. Although clinical disease in quokkas due to MaHV-6 infection has not been described, skin lesions in the form of ulcers (oral and cloacal), respiratory signs, and ocular signs (e.g. conjunctivitis) may be present, as these signs have all been observed in animals positive to MaHV-1, -2, -3, and -4. Spill-over of MaHV-6 may present fatal to other species.

- Tracking the incidence, prevalence and population abundance of flower cells and Atly in quokkas on Rottnest Island is considered by this project to be a top and urgent priority for the Rottnest Island Authority and the Department of Parks and Wildlife. Given the absence of conclusive evidence indicating that either flower cells are or are not neoplastic in nature and represent a pre-leukaemic state in Rottnest Island quokkas, further studies are critical to answer what flower cells are and represent for quokkas. See section 8.7 for the studies proposed for this task.
- Given that low protein diets have been previously linked to anaemia, replanting on Rottnest Island could be directed towards high protein food plants (requiring nutritional analyses of food plant species).
- Movement of animals from Rottnest Island to the mainland or vice versa is unadvisable, and should not be considered for various reasons. First, if a retrovirus is responsible for the presence of flower cells and Atly, the risk of animal-to-animal infection must be considered. Although *Salmonella* spp. infections can be treated if needed, the high abundance and richness of *Salmonella* spp. on Rottnest Island may present a serious problem for mainland animals, who may be immunologically naïve to it. However, immunological testing would be necessary to confirm this. Although *C. neoformans* var. *grubii* is not transmitted by direct contact, it is not known to what degree mainland animals are susceptible to this yeast.
- Disease surveillance and health monitoring should include complete haematology and blood chemistry studies (including vitamin E), which are the only tools that would enable tracking of clinical and subclinical disease in quokkas caused by any infectious organism. This course of action would require of an interdisciplinary approach led by veterinary personnel, with the support of professionals from animal conservation fields such as ecology, biology and zoology among others. Additional resources and procedures needed to carry out disease surveillance and health monitoring of quokkas may include transportation, anaesthesia, physical examination equipment, biological sampling reagents, and access to laboratory facilities and reagents needed to carry out the necessary tests (e.g. PCR, culturing, incubators, laminar flow cabinets, microscopes).
- Changes in prevalence, distribution, incidence, presence or absence of disease among other aspects, are necessary to plan management strategies and an emergency response plan. Although this is the right course of action for both mainland and island populations, it is strongly advisable that Rottnest Island Authority continues strict and uninterrupted monitoring of *Salmonella* spp., *C.*

neoformans var. *grubii*, MaHV-6, microfilariae and atypical lymphocyte populations in the quokka.

8.7 Future Directions of Research

- Ongoing disease surveillance and health monitoring of wild quokkas should include individuals from other subpopulations, i.e. Bald Island and Stirling Range.
- Further comparison and analysis of the health and disease status of mainland and island subpopulations, would benefit of greater sample sizes, and a comparable representation of males and females for the mainland sample. This would provide greater sensitivity of the statistical analyses necessary to compare both cohorts (i.e. island and mainland).
- Determine the environmental source or sources for *C. neoformans* var. *grubii* on Rottnest Island, and assess and establish the epidemiological risk of infection in humans.
- Determine latex cryptococcal agglutination antigen test (LCAT) titres for clinical and subclinical disease in quokkas on Rottnest Island for clinical management of cryptococcosis.
- Determine the microfilariae species present in quokkas on Rottnest Island via PCR and sequencing, and investigate the effect of microfilariae on the immune system of the animals through in vitro stimulation of monocytes and macrophages with microfilarial lysate. Production of cytokines such as IFN-γ, IL-10 and IL-13 could be considered.
- Confirm that flower cells and Atly morphologies in animals on Rottnest Island are lymphocytes, through immunophenotyping using flow cytometry and immunohistochemistry. Similarly, carry out cytological evaluation of bone marrow aspirates (biopsies). This may improve the understanding of the pathophysiology of these cells and possibly define their clinical significance (if any). Establish the presence or absence of a retroviral infection (specifically a deltaretrovirus) through molecular studies (e.g. RNA sequencing) and carry out its corresponding phylogenetic analysis. If detected, further studies should aim to isolate in cell culture and further characterise this virus as well as establish the extent of distribution, determine transmission and infectivity.
- Isolate MaHV-6 to allow its cytopathic effect to be defined. Also, designing a serology test (e.g. a serum neutralisation test) to screen for antibodies against

MaHV-6. This would provide the opportunity to screen other species sharing the same ecological niche as the quokka.

- Catecholamines (CCs) as well as glucocorticoids (GCs) assays should be considered as part of the health screening of wild quokkas. A variety of samples could be used for these assays. If HMT and BLC studies are being carried out, then measurement of both CCs (adrenaline and noradrenaline) and GCs (cortisol) is advisable, and blood would be the sample of choice (Sheriff *et al.* 2011). Non-invasive methods also exist and are used to measure GCs, this include saliva, urine, faeces and hair (Sheriff *et al.* 2011). Similarly, stress surveillance, understood as the ongoing tracking of levels of stress in free-ranging quokkas in combination with infection parameters (Hing *et al.* 2016), would facilitate a better understanding of how these factors interrelate and affect their health, provided the proper multivariate correlational analyses are carried out (i.e. environmental variables), In this context, given that all major organisms examined by this study (e.g. *Salmonella* spp., *C. neoformans* var. *grubii*, MaHV-6) are known to cause disease in the host when immunosuppressed, which is a possible outcome of chronic stress, stress surveillance is advisable.

8.8 Conclusion

This thesis not only provides new insight into the diseases and health of wild quokkas, but also presents updated data for disease aspects in this species. The epidemiological data obtained (e.g. prevalence and distribution), has a high reliability factor considering the robust sample size used, and may be incorporated in future risk analysis planning. This thesis also presents the first reference intervals for haematology, blood chemistry and vitamin E, of quokkas on Rottnest Island and selected locations on the mainland, which would aid in the assessment of the overall health of wild individuals by providing a baseline against which any changes can be measured. Overall, the outcomes of this project have highlighted the need for continuous disease and health surveillance, and have provided evidence of the importance of wildlife health professionals in the conservation of Australian native wildlife. Ongoing surveillance of infectious diseases and health monitoring in *S. brachyurus* is essential to a sound approach to conservation of this vulnerable species.

9. References

Aaskov, J., & Doherty, R. 1994. Arboviral zoonoses of Australasia. In *Handbook of Zoonoses. Section B: Viral*, edited by A. S. Benenson, T. F. Tsai, F. Fenner & M. Torten. 2nd ed. Boca Raton: CRC Press. 289-306.

Abbott, I. 2001. Aboriginal names of mammal species in south-west Western Australia. *CALMScience* 3 (4):433-486.

Abbott, I. 2006. Mammalian faunal collapse in Western Australia, 1875-1925: The hypothesised role of epizootic disease and a conceptual model of its origin, introduction, transmission, and spread. *Australian Zoologist* 33 (4):530-561.

Abbott, I. 2008. Historical perspectives of the ecology of some conspicuous vertebrates species in south-west Western Australia. *Conservation Science Western Australia* 6 (3):1-214.

Ackermann, M. 2006. Pathogenesis of gammaherpesvirus infections. *Veterinary Microbiology* 113 (3-4):211-222.

Acland, H.M. 1981. Parma wallaby herpesvirus infection. *Journal of Wildlife Diseases* 17 (3):471-477.

Adkesson, M.J., Gorman, M.E., Hsiao, V., Whittington, J.K., & Langan, J.N. 2007. *Toxoplasma gondii* inclusions in peripheral blood leukocytes of a red-necked wallaby (*Macropus rufogriseus*). *Veterinary Clinical Pathology* 36 (1):97-100.

Aguirre, A.A., & Tabor, G.M. 2008. Global factors driving emerging infectious diseases: Impact on wildlife populations. In *Annals of the New York Academy of Sciences*, edited by O. A. E. Sparagano, J. C. Maillard & J. V. Figueroa.

Alacs, E., Spencer, P., de Tores, P., & Krauss, S. 2011. Population genetic structure of island and mainland populations of the quokka, *Setonix brachyurus* (Macropodidae): a comparison of AFLP and microsatellite markers. *Conservation Genetics* 12 (1):297-309.

Algar, D., Angus, G.J., & Onus, M.L. 2011. Eradication of feral cats on Rottnest Island, Western Australia. *Journal of the Royal Society of Western Australia* 94 (3):439-443.

Alley, M.R., Hale, K.A., Cash, W., Ha, H.J., & Howe, L. 2010. Concurrent avian malaria and avipox virus infection in translocated South Island saddlebacks (*Philesturnus carunculatus carunculatus*). *New Zealand Veterinary Journal* 58 (4):218-223.

Allison, R.W. 2012. Laboratory Evaluation of the Liver. In *Veterinary Hematology And Clinical Chemistry*, edited by T. Mary Anna, W. Glade, W. A. Robin & W. C. Terry. 2nd Ed. ed. Ames, Iowa: Wiley-Blackwell. 401-424.

Altman, D.G. 1999. *Practical Statistics for Medical Research*. Boca Raton, Fla.: Chapman & Hall/CRC.

Amaddeo, D., Cardeti, G., & Autorino, G.L. 1995. Isolation of encephalomyocarditis virus from dormice (*Myoxus glis*) in Italy. *Journal of Wildlife Diseases* 31 (2):238-242.

Amavisit, P., Browning, G.F., Lightfoot, D., Church, S., Anderson, G.A., Whithear, K.G., & Markham, P.F. 2001. Rapid PCR detection of *Salmonella* in horse faecal samples. *Veterinary Microbiology* 79 (1):63-74.

Amery-Gale, J., Vaz, P.K., Whiteley, P., Tatarczuch, L., Taggart, D.A., Charles, J.A., Schultz, D., Ficorilli, N.P., Devlin, J.M., & Wilks, C.R. 2014. Detection and identification of a gammaherpesvirus in *Antechinus* spp. in Australia. *Journal of Wildlife Diseases* 50 (2):334-339.

Anderson, M.J. 2001. A new method for non-parametric multivariate analysis of variance. *Austral Ecology* 26 (1):32-46.

Anderson, R.J., House, J.K., Smith, B.P., Kinde, H., Walker, R.L., Vande Steeg, B.J., & Breitmeyer, R.E. 2001. Epidemiologic and biological characteristics of salmonellosis in three dairy herds. *Journal of the American Veterinary Medical Association* 219 (3):310-322.

Animal Health Australia. 2011. Disease Strategy: Equiine Influenza (Version 3.1). Australian Veterinary Emergency Plan (AUSVETPLAN) edition 3. Canberra, ACT. Primary Industries Ministerial Council. [accessed 21st March, 2015]. Available from http://www.animalhealthaustralia.com.au/programs/emergency-animal-disease-preparedness/ausvetplan/disease-strategies/.

Appleby, E.C. 1969. Tumours in captive wild animals: some observations and comparisons. *Acta Zoologica et Pathologica Antverpiensia* 48:77-92.

Arundel, J.H., Barker, I.K., & Beveridge, I. 1977. Diseases of marsupials. In *The Biology of Marsupials*, edited by B. Stonehouse & D. Gilmore. London: The Macmillan Press Ltd. 141-154.

Arundel, J.H., Beveridge, I., & Presidente, P.J. 1979. Parasites and pathological findings in enclosed and free-ranging populations of *Macropus rufus* (Demarest) (Marsupialia) at Menindee, New South Wales. *Wildlife Research* 6 (3):361-379.

Ashman, R.B., Holmes, R.M., & Keast, D. 1977. The effect of neonatal thymectomy on the ontogeny of mitogen responses in the quokka (*Setonix brachyurus*). *Developmental and Comparative Immunology* 1 (1):47-57.

Atkins, B.L., & Gottlieb, T. 2014. Skin and soft tissue infections caused by nontuberculous mycobacteria. *Current Opinion in Infectious Diseases* 27 (2):137-145.

Atkinson, C.T., & LaPointe, D.A. 2009. Introduced avian diseases, climate change, and the future of Hawaiian honeycreepers. *Journal of Avian Medicine and Surgery* 23 (1):53-63.

Atlas of Living Australia. 2013. Australian Government [accessed 20th February 2014]. Available from http://www.ala.org.au/.

Attwood, H.D., & Woolley, P.A. 1973. Spontaneous malignant neoplasms in dasyurid marsupials. *Journal of Comparative Pathology* 83 (4):569-581.

Attwood, H.D., Woolley, P.A., & Rickard, M.D. 1975. Toxoplasmosis in dasyurid marsupials. *Journal of Wildlife Diseases* 11 (4):543-551.

Austen, J.M., Friend, J.A., Yang, R., & Ryan, U.M. 2014. Further characterisation of two Eimeria species (*Eimeria quokka* and *Eimeria setonicis*) in quokkas (*Setonix brachyurus*). *Experimental Parasitology* 138 (1):48-54.

Austen, J.M., Jefferies, R., Friend, J.A., Ryan, U., Adams, P., & Reid, S.A. 2009. Morphological and molecular characterization of *Trypanosoma copemani* n. sp. (Trypanosomatidae) isolated from Gilbert's potoroo (*Potorous gilbertii*) and quokka (*Setonix brachyurus*). *Parasitology* 136 (7):783-792.

Austen, J.M., Ryan, U.M., Friend, J.A., Ditcham, W.G.F., & Reid, S.A. 2011. Vector of *Trypanosoma copemani* identified as *Ixodes* sp. *Parasitology* 138 (7):866-872.

Australian Bureau of Meteorology. 2012. *Climate statistics for Australian locations*. Australian Government, Monday, July 9th [accessed Tuesday, July 10th 2012]. Available from http://www.bom.gov.au/climate/averages/tables/cw_009193.shtml.

Australian Veterinary Association. 2013. *Policy Compendium, policy 5.1: Electronic Identification on Animals*. Australian Veterinary Association [accessed September 10th 2013]. Available from http://www.ava.com.au.

Australian Wildlife Health Network. 2011. Coccidiosis in kangaroos. Australian Wildlife Health Network. [accessed 19th May, 2014]. Available from https://wildlifehealthaustralia.com.au/FactSheets.aspx.

Averis, S., Thompson, R.C., Lymbery, A.J., Wayne, A.F., Morris, K.D., & Smith, A. 2009. The diversity, distribution and host-parasite associations of trypanosomes in Western Australian wildlife. *Parasitology* 136 (11):1269-1279.

Bagcigil, A.F., Ikiz, S., Dokuzeylul, B., Basaran, B., Or, E., & Ozgur, N.Y. 2007. Fecal shedding of *Salmonella* spp. in dogs. *Journal of Veterinary Medical Science* 69 (7):775-777.

Bagnall, B.G., & Wilson, G.R. 1974. Molluscum contagiosum in a red kangaroo. *Australasian Journal of Dermatology* 15 (3):115-120.
Bain, K., Wayne, A., & Bencini, R. 2015. Risks in extrapolating habitat preferences over the geographical range of threatened taxa: a case study of the quokka (*Setonix brachyurus*) in the southern forests of Western Australia. *Wildlife Research* 42 (4):334.
Barker, I.K., Harrigan, K.E., & Dempster, J.K. 1972. Coccidiosis in wild grey kangaroos. *International Journal for Parasitology* 2 (2):187-192.
Barker, I.K., O'Callaghan, M.G., & Beveridge, I. 1988a. Eimeria spp. (Apicomplexa:Eimeriidae) parasitic in the rat-kangaroos *Hypsiprymnodon moschatus*, *Potorous tridactylus*, *Aepyprymnus rufescens* and *Bettongia gaimardi* (Marsupialia:Potoroidae). *International Journal for Parasitology* 18 (7):947-953.
Barker, I.K., O'Callaghan, M.G., & Beveridge, I. 1988b. Eimeria spp. (Apicomplexa:Eimeriidae) parasitic in wallabies and kangaroos of the genera *Setonix*, *Thylogale*, *Wallabia*, *Lagorchestes* and *Dendrolagus* (Marsupialia: Macropodidae). *International Journal for Parasitology* 18 (7):955-962.
Barker, I.K., O'Callaghan, M.G., & Beveridge, I. 1989. Host-parasite associations of *Eimeria* spp. (Apicomplexa:Eimeriidae) in kangaroos and wallabies of the genus *Macropus* (Marsupialia:Macropodidae). *International Journal for Parasitology* 19 (3):241-263.
Barker, S. 1961. Studies on marsupial nutrition III. The copper-molybdenum-inorganic sulphate interaction in the Rottnest quokka *Setonix brachyurus* (Quoy & Gaimard). *Australian Journal of Biological Sciences* 14 (4):646-658.
Barker, S., Glover, R., Jacobsen, P., & Kakulas, B.A. 1974. Seasonal anaemia in the rottnest quokka, *Setonix brachyurus* (Quoy & Gaimard) (Marsupialia; Macropodidae). *Comparative Biochemistry and Physiology Part A: Physiology* 49 (1):147-157.
Barnes, T.S., Goldizen, A.W., & Coleman, G.T. 2008. Hematology and serum biochemistry of the brush-tailed rock-wallaby (*Petrogale penicillata*). *Journal of Wildlife Diseases* 44 (2):295-303.
Barrandeguy, M., Vissani, A., Olguin, C., Becerra, L., Mino, S., Pereda, A., Oriol, J., & Thiry, E. 2008. Experimental reactivation of equine herpesvirus-3 following corticosteroid treatment. *Equine Veterinary Journal* 40 (6):593-595.
Barrow, P.A. 2012. Salmonellosis. In *The Merck Veterinary Manual Online*, ed S. E. Aiello & M. A. Moses. Whitehouse Station, USA: Merck Sharp & Dohme Corp. [cited August 13th 2013] Available from http://www.merckmanuals.com/vet/digestive_system/salmonellosis/overview_of_salmonellosis.html?qt=salmonellosis&alt=sh.
Barrow, P.A., Jones, M.A., & Thomson, N. 2010. Salmonella. In *Pathogenesis of Bacterial Infections in Animals*, edited by C. L. Gyles, J. F. Prescott, G. Songer & C. O. Thoen. 4th ed. Ames: Wiley-Blackwell. 231-266.
Bartholomew, G.A. 1956. Temperature regulation in the macropod marsupial, *Setonix brachyurus*. *Physiological Zoology* 29 (1):26-40.
Bauer, A.W., Kirby, W.M., Sherris, J.C., & Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology* 45 (4):493-496.
Baumgarth, N., Herman, O.C., Jager, G.C., Brown, L., Herzenberg, L.A., & Herzenberg, L.A. 1999. Innate and acquired humoral immunities to influenza virus are mediated by distinct arms of the immune system. *Proceedings of the National Academy of Sciences* 96 (5):2250-2255.
Bauwens, L., Vercammen, F., Wuytack, C., Van Looveren, K., & Swinne, D. 2004. Isolation of *Cryptococcus neoformans* in Antwerp Zoo's nocturnal house. *Mycoses* 47 (7):292-296.
Beemer, A.M., Schneerson-Porat, S., & Kuttin, E.S. 1970. Rhodotorula mucilaginosa dermatitis on feathered parts of chickens: an epizootic on a poultry farm. *Avian Diseases* 14 (2):234-239.

Behnke, J.M., Sinski, E., & Wakelin, D. 1999. Primary infections with *Babesia microti* are not prolonged by concurrent *Heligmosomoides polygyrus*. *Parasitology International* 48 (2):183-187.
Bellehumeur, C., Lair, S., Romero, C.H., Provost, C., Nielsen, O., & Gagnon, C.A. 2015. Identification of a novel herpesvirus associated with a penile proliferative lesion in a beluga (*Delphinapterus leucas*). *Journal of Wildlife Diseases* 51 (1):244-249.
Bennett, M.D., & Hobbs, R.P. 2011. A new eimeria species parasitic in *Isoodon obesulus* (Marsupialia: Peramelidae) in Western Australia. *Journal of Parasitology* 97 (6):1129-1131.
Bennett, M.D., Woolford, L., O'Hara, A.J., Nicholls, P.K., & Warren, K.S. 2008. Clinical chemistry values and tissue enzyme activities in western barred bandicoots (*Perameles bougainville*). *Veterinary Clinical Pathology* 37 (2):221-224.
Bennett, M.D., Woolford, L., O'Hara, A.J., Nicholls, P.K., Warren, K.S., Hulme-Moir, K.L., & Clark, P. 2007. Hematologic characteristics of captive western barred bandicoots (*Perameles bougainville*) from Western Australia. *Veterinary Clinical Pathology* 36 (4):348-353.
Bennett, M.D., Woolford, L., Ohara, A.J., Nicholls, P.K., Warren, K., & Hobbs, R.P. 2006. A new eimeria species parasitic in western barred bandicoots, *Perameles bougainville* (Marsupialia: Peramelidae), in western Australia. *Journal of Parasitology* 92 (6):1292-1294.
Bensley, B.A. 1903. III. On the Evolution of the Australian Marsupialia; with Remarks on the Relationships of the Marsupials in general. *Transactions of the Linnean Society of London. 2nd Series: Zoology* 9 (3):83-217.
Bergeron, N., Corriveau, J., Letellier, A., Daigle, F., Lessard, L., & Quessy, S. 2009. Interaction between host cells and septicemic Salmonella enterica serovar typhimurium isolates from pigs. *Journal of Clinical Microbiology* 47 (11):3413-3419.
Bermúdez, R., Faílde, L.D., Losada, A.P., Nieto, J.M., & Quiroga, M.I. 2009. Toxoplasmosis in Bennett's wallabies (*Macropus rufogriseus*) in Spain. *Veterinary Parasitology* 160 (1-2):155-158.
Berry, W.L., Jardine, J.E., & Espie, I.W. 1997. Pulmonary cryptococcoma and cryptococcal meningoencephalomyelitis in a king cheetah (*Acinonyx jubatus*). *Journal of Zoo and Wildlife Medicine* 28 (4):485-490.
Bettiol, S.S., Jakes, K., Le, D.D., Goldsmid, J.M., & Hocking, G. 1998. First record of trypanosomes in Tasmanian bandicoots. *Journal of Parasitology* 84 (3):538-541.
Bettiol, S.S., Obendorf, D.L., Nowarkowski, M., & Goldsmid, J.M. 2000. Pathology of experimental toxoplasmosis in eastern barred bandicoots in Tasmania. *Journal of Wildlife Diseases* 36 (1):141-144.
Beveridge, I. 1993. Marsupial Parasitic Diseases. In *Zoo and Wild Animal Medicine. Current Therapy 3*, edited by M. E. Fowler. Philadelphia: WB Saunders. 288-293.
Bicknese, E.J., Childress, A.L., & Wellehan Jr, J.F.X. 2010. A novel herpesvirus of the proposed genus chelonivirus from an asymptomatic bowsprit tortoise (*Chersina angulata*). *Journal of Zoo and Wildlife Medicine* 41 (2):353-358.
Biet, F., Boschiroli, M.L., Thorel, M.F., & Guilloteau, L.A. 2005. Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium*-intracellulare complex (MAC). *Veterinary Research* 36 (3):411-436.
Billiards, S.S., King, J.M., & Agar, N.S. 1999. Comparative erythrocyte metabolism in three species of marsupials from Western Australia. *Comparative Haematology International* 9 (2):86-91.
Billingham, R.E. 1986. Immunologic advantages and disadvantages of being a female. In *Reproductive Immunology*, edited by D. A. Clark & B. A. Croy. New York: Elsevier. 1-9.
Billinis, C. 2009. Encephalomyocarditis virus infection in wildlife species in Greece. *Journal of Wildlife Diseases* 45 (2):522-526.
Birdlife Australia. 2015. *Rock Dove* [accessed 25th August, 2015. Available from http://birdlife.org.au/bird-profile/Rock-Dove.

Black, P.H. 1994. Central nervous system-immune system interactions: psychoneuroendocrinology of stress and its immune consequences. *Antimicrobial Agents and Chemotherapy* 38 (1):1-6.
Bleasdale, B., Lott, P.J., Jagannathan, A., Stevens, M.P., Birtles, R.J., & Wigley, P. 2009. The *Salmonella* pathogenicity island 2-encoded type III secretion system is essential for the survival of *Salmonella enterica* serovar typhimurium in free-living amoebae. *Applied and Environmental Microbiology* 75 (6):1793-1795.
Blumstein, D.T., Daniel, J.C., & McLean, I.G. 2001. Group size effects in quokkas. *Australian Journal of Zoology* 49 (6):641-649.
Blyde, D. 1994. Management and diseases of macropods. In *Wildlife: The T.G. Hungerford Refresher Course for Veterinarians*, *Dubbo*, *19-23 September 1994*, 247-251: Postgraduate Committee in Veterinary Science. University of Sydney.
Blyde, D. 1999. Advances in treating diseases of macropods. In *Australian Wildlife. Healthcare and Management*, *Dubbo*, *13-17 September 1999*, 439-452: Postgraduate Foundation in Veterinary Science. University of Sydney.
Bolliger, A., & Macindoe, N.M. 1950. Eye changes in a marsupial experimentally infected with kala-azar and trypanosomiasis. *American Journal of Ophthalmology* 33 (12):1871-1877.
Bonney, K.R., & Wynne, C.D.L. 2004. Studies of learning and problem solving in two species of Australian marsupials. *Neuroscience & Biobehavioral Reviews* 28 (6):583-594.
Bordes, F., & Morand, S. 2011. The impact of multiple infections on wild animal hosts: a review. Infection Ecology & Epidemiology 1. [accessed. Available from http://www.ncbi.nlm.nih.gov/pubmed/22957114.
Botero, A., Thompson, C.K., Peacock, C.S., Clode, P.L., Nicholls, P.K., Wayne, A.F., Lymbery, A.J., & Thompson, R.C. 2013. Trypanosomes genetic diversity, polyparasitism and the population decline of the critically endangered Australian marsupial, the brush tailed bettong or woylie (*Bettongia penicillata*). *International Journal for Parasitology: Parasites and Wildlife* 2:77-89.
Bourges-Abella, N., Geffre, A., Concordet, D., Braun, J.P., & Trumel, C. 2011. Canine reference intervals for the Sysmex XT-2000iV hematology analyzer. *Veterinary Clinical Pathology* 40 (3):303-315.
Boyd, A.M., Hall, R.A., Gemmell, R.T., & Kay, B.H. 2001. Experimental infection of Australian brushtail possums, *Trichosurus vulpecula* (Phalangeridae: Marsupialia), with Ross River and Barmah Forest viruses by use of a natural mosquito vector system. *American Journal of Tropical Medicine and Hygiene* 65 (6):777-782.
Brady, M.T., O'Neill, S.M., Dalton, J.P., & Mills, K.H.G. 1999. Fasciola hepatica suppresses a protective Th1 response against *Bordetella pertussis*. *Infection and Immunity* 67 (10):5372-5378.
Brandt, J.H., Diolo, M., Hassanin, A., Melville, R.A., Olson, L.E., Seveau, A., & Timm, R.M. 2001. Debate on the authenticity of *Pseudonovibos spiralis* as a new species of wild bovid from Vietnam and Cambodia: Debate on the authenticity of *Pseudonovibos spiralis*. *Journal of Zoology* 255 (4):437-444.
Bray, J.R., & Curtis, J.T. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs* 27 (4):326-349.
Bray, M. 2011. Cross-Species Transmission Of Poxviruses. In *New and Evolving Infections of the 21st Century*, edited by I. W. Fong & K. Alibek. New York: Springer-Verlag. 129-159.
Brearley, G., Rhodes, J., Bradley, A., Baxter, G., Seabrook, L., Lunney, D., Liu, Y., & McAlpine, C. 2013. Wildlife disease prevalence in human-modified landscapes. *Biological Reviews of the Cambridge Philosophical Society* 88 (2):427-442.
Breed, A.C. 2007. Parasites and disease. In *Mice and Rats of Australia*, edited by W. Breed & F. Ford. Melbourne: CSIRO. 139-148.
Brenner, F.W., Villar, R.G., Angulo, F.J., Tauxe, R., & Swaminathan, B. 2000. Salmonella nomenclature. *Journal of Clinical Microbiology* 38 (7):2465-2467.

Brereton, R., Bennett, S., & Mansergh, I. 1995. Enhanced greenhouse climate change and its potential effect on selected fauna of south-eastern Australia: A trend analysis. *Biological Conservation* 72 (3):339-354.
Brigelius-Flohé, R., & Traber, M.G. 1999. Vitamin E: function and metabolism. *FASEB Journal* 13 (10):1145-1155.
Briggs, C., Longair, I., Slavik, M., Thwaite, K., Mills, R., Thavaraja, V., Foster, A., Romanin, D., & MacHin, S.J. 2009. Can automated blood film analysis replace the manual differential? An evaluation of the CellaVision DM96 automated image analysis system. *International Journal of Laboratory Hematology* 31 (1):48-60.
Briles, D.E., Nahm, M., Schroer, K., Davie, J., Baker, P., Kearney, J., & Barletta, R. 1981. Antiphosphocholine antibodies found in normal mouse serum are protective against intravenous infection with type 3 *Streptococcus pneumoniae*. *The Journal of Experimental Medicine* 153 (3):694-705.
Britt Jr, J.O., Frost, D.F., & Cockrill, J.M. 1994. Fatal herpesviral hepatitis in a red kangaroo (*Macropus rufus*). *Journal of Zoo and Wildlife Medicine* 25 (4):580-584.
Brock, T.D. 1999. Robert Koch: A Life in Medicine and Bacteriology: ASM Press. http://www.asmscience.org/content/book/10.1128/9781555818272 (accessed 20th May, 2015).
Brown, L.D., Cai, T.T., & DasGupta, A. 2001. Interval estimation for a binomial proportion. *Statistical Science* 16 (2):101-117.
Bryant, G.L., Fleming, P.A., Twomey, L., & Warren, K.A. 2012. Factors affecting hematology and plasma biochemistry in the southwest Carpet Python (*Morelia spilota imbricata*). *Journal of Wildlife Diseases* 48 (2):282-294.
Buddle, B.M., & Young, L.J. 2000. Immunobiology of mycobacterial infections in marsupials. *Developmental and Comparative Immunology* 24 (5):517-529.
Burbidge, A.A., & McKenzie, N.L. 1989. Patterns in the modern decline of western Australia's vertebrate fauna: Causes and conservation implications. *Biological Conservation* 50 (1-4):143-198.
Burmeister, T., Schwartz, S., Hummel, M., Hoelzer, D., & Thiel, E. 2007. No genetic evidence for involvement of Deltaretroviruses in adult patients with precursor and mature T-cell neoplasms. *Retrovirology* 4.
Burmeister, T., Schwartz, S., & Thiel, E. 2001. A PCR primer system for detecting oncoretroviruses based on conserved DNA sequence motifs of animal retroviruses and its application to human leukaemias and lymphomas. *Journal of General Virology* 82 (9):2205-2213.
Burrows, N.D., Ward, B., & Robinson, A.D. 1995. Jarrah forest fire history from stem analysis and anthropological evidence. *Australian Forestry* 58 (1):7-16.
Buxton, D., & Maley, S.W. 2013. Toxoplasmosis. In *OIE Terrestrial Manual*: OIE Available from http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/.
Cafarchia, C. 2012. Yeast Infections. In *Infectious Diseases of Wild Mammals and Birds in Europe*, edited by D. Gavier-Widén, J. P. Duff & A. Meredith. Chichester: Wiley-Blackwell. 462-465.
Caley, P., Hickling, G.J., Cowan, P.E., & Pfeiffer, D.U. 1999. Effects of sustained control of brushtail possums on levels of *Mycobacterium bovis* infection in cattle and brushtail possum populations from Hohotaka, New Zealand. *New Zealand Veterinary Journal* 47 (4):133-142.
Callinan, I. 2008. Equine influenza the August 2007 outbreak in Australia : report of the Equine Influenza Inquiry. Canberra, ACT: Department of Agriculture. Australian Government. [accessed 21st March, 2015]. Available from http://trove.nla.gov.au/version/30659476.
Callinan, R.B., & Kefford, B. 1981. Mortalities associated with herpesvirus infection in captive macropods. *Journal of Wildlife Diseases* 17 (2):311-317.
Canfield, P.J., & Hartley, W.J. 1992. A survey and review of hepatobiliary lesions in Australian macropods. *Journal of Comparative Pathology* 107 (2):147-167.

Canfield, P.J., Hartley, W.J., & Dubey, J.P. 1990. Lesions of toxoplasmosis in Australian marsupials. *Journal of Comparative Pathology* 103 (2):159-167.
Canfield, P.J., Hartley, W.J., & Reddacliff, G.L. 1990a. Spontaneous proliferations in Australian marsupials - A survey and review. 1. Macropods, koalas, wombats, possums and gliders. *Journal of Comparative Pathology* 103 (2):135-146.
Canfield, P.J., Hartley, W.J., & Reddacliff, G.L. 1990b. Spontaneous proliferations in Australian marsupials - A survey and review. 2. Dasyurids and bandicoots. *Journal of Comparative Pathology* 103 (2):147-158.
Canteros, C.E., Rodero, L., Rivas, M.C., & Davel, G. 1996. A rapid urease test for presumptive identification of *Cryptococcus neoformans*. *Mycopathologia* 136 (1):21-23.
Cardillo, M., Mace, G.M., Gittleman, J.L., & Purvis, A. 2006. Latent extinction risk and the future battlegrounds of mammal conservation. *Proceedings of the National Academy of Sciences of the United States of America* 103 (11):4157-4161.
Carocci, M., & Bakkali-Kassimi, L. 2012. The encephalomyocarditis virus. *Virulence* 3 (4):351-367.
Carver, S., Bestall, A., Jardine, A., & Ostfeld, R.S. 2009. Influence of hosts on the ecology of arboviral transmission: Potential mechanisms influencing dengue, Murray Valley encephalitis, and Ross River virus in Australia. *Vector-Borne and Zoonotic Diseases* 9 (1):51-64.
Cary, G.J. 2002. Importance of a Changing Climate For Fire Regimes In Australia. In *Flammable Australia: Fire Regimes and Biodiversity of a Continent*, edited by R. A. Bradstock, J. E. Williams & A. M. Gill. Melbourne: Cambridge University Press. 26-49.
Casadevall, A., & Perfect, J.R. 1998. *Cryptococcus neoformans*. Washington D.C.: American Society for Microbiology.
Cattet, M.R., Christison, K., Caulkett, N.A., & Stenhouse, G.B. 2003. Physiologic responses of grizzly bears to different methods of capture. *Journal of Wildlife Diseases* 39 (3):649-654.
Caughley, G., & Gunn, A. 1996. *Conservation Biology in Theory and Practice*. Massachusetts: Blackwell Science.
Ceballos, G., & Ehrlich, P.R. 2002. Mammal population losses and the extinction crisis. *Science* 296 (5569):904-907.
Chang, S.T., Hsieh, Y.C., Lu, Y.H., Tzeng, C.C., Lin, C.N., & Chuang, S.S. 2008. Floral leukemic cells transformed from marginal zone lymphoma. *Pathology Research and Practice* 204 (1):23-26.
Chapman, S.R. 1999. Mycophagy by quokka (*Setonix brachyurus*) on Rottnest Island and the relationship between trees, fungal spores, scats and spore dispersal: comparison to Dwellingup population. Hons. diss., School of Biological Sciences, Murdoch University, Perth.
Chappell, L., Kaiser, P., Barrow, P., Jones, M.A., Johnston, C., & Wigley, P. 2009. The immunobiology of avian systemic salmonellosis. *Veterinary Immunology and Immunopathology* 128 (1-3):53-59.
Chauvet, M.T., Colne, T., Hurpet, D., Chauvet, J., & Acher, R. 1983. Marsupial neurohypophysial hormones: Identification of mesotocin, lysine vasopressin, and phenypressin in the quokka wallaby (*Setonix brachyurus*). *General and Comparative Endocrinology* 51 (2):309-315.
Chen, S., Sorrell, T., Nimmo, G., Speed, B., Currie, B., Ellis, D., Marriott, D., Pfeiffer, T., Parr, D., Byth, K., Allworth, A., Beaman, M., Benn, R., Collignon, P., Ellis-Pegler, R., Ferguson, J., Fisher, D., Faoagali, J., Forgan-Smith, R., Fuller, A., Gollege, C., Gordon, D., Gosbell, I., Gottlieb, T., Heath, C., Hoy, J., Hume, S., Jones, P., Kelly, R., Kocuiba, K., Kotsiou, G., Lang, S., LeBrooy, J., Lum, G., McAleer, R., McCormack, J., Merrell, H., Mijch, A., Muir, D., Munro, R., Newton, P., Ott, K., Papanoum, K., Pritchard, R., Pearman, J., Reiss-Levy, E., Robson, J., Schooneveldt, J., Shaw, D., Sinickas, V., Slavin, M., Tilse, M., Tosolini, F., Whitby, M., & Wood, G. 2000. Epidemiology and host- and variety-dependent characteristics of infection due to *Cryptococcus*

neoformans in Australia and New Zealand. *Clinical Infectious Diseases* 31 (2):499-508.
Choo, S.W., Dutta, A., Wong, G.J., Wee, W.Y., Ang, M.Y., & Siow, C.C. 2016. Comparative genomic analysis reveals a possible novel non-tuberculous *Mycobacterium* species with high pathogenic potential. *PLoS One* 11 (4):e0150413.
Chosewood, L.C., & Wilson, D.E. 2009. *Biosafety in microbiological and biomedical laboratories*: US Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institutes of Health.
Christensen, W.B. 1946. Urea decomposition as a means of differentiating proteus and paracolon cultures from each other and from salmonella and shigella types. *Journal of Bacteriology* 52 (4):461-466.
Chrousos, G.P. 2010. Stress and sex versus immunity and inflammation. *Science Signaling* 3 (143).
Citino, S.B., Homer, B.L., Gaskin, J.M., & Wickham, D.J. 1988. Fatal Encephalomyocarditis Virus Infection in a Sumatran Orangutan (*Pongo pygmaeus abelii*). *Journal of Zoo Animal Medicine* 19 (4):214-218.
Clark, E.C., Silver, S.M., Hollick, G.E., & Rinaldi, M.G. 1995. Continuous ambulatory peritoneal dialysis complicated by *Aureobasidium pullulans* peritonitis. *American Journal of Nephrology* 15 (4):353-355.
Clark, P. 2004. *Haematology of Australian Mammals*. Collingwood: CSIRO PUBLISHING.
Clark, P. 2006. Haematological characteristics of morbid members of the Macropodidae. *Comparative Clinical Pathology* 14 (4):191-196.
Clark, P., Holz, P., Booth, R., Jakob-Hoff, R., Cooper, D.W., Boardman, W.S., Parry, B.W., & Norman, R.J. 2003. Haematological characteristics of captive Parma wallabies (*Macropus parma*). *Comparative Clinical Pathology* 12 (1):11-16.
Clark, P., & Spencer, P.B.S. 2006. Haematological characteristics of wild quokka (*Setonix brachyurus*). *Comparative Clinical Pathology* 15 (2):82-86.
Clark, P., & Spencer, P.B.S. 2007. Description of three new species of *Theileria bettencourt*, franca & borges, 1907 from macropodoidea in Western Australia. *Transactions of the Royal Society of South Australia* 131 (1):100-106.
Clarke, J., Warren, K., Calver, M., de Tores, P., Mills, J., & Robertson, I. 2013. Hematologic and serum biochemical reference ranges and an assessment of exposure to infectious diseases prior to translocation of the threatened western ringtail possum (*Pseudocheirus occidentalis*). *Journal of Wildlife Diseases* 49 (4):831-840.
Clarke, K.R. 1993. Non-parametric multivariate analyses of changes in community structure. *Australian Journal of Ecology* 18 (1):117-143.
Clarke, P., & Tyler, K.L. 2010. Reoviruses: General Features. In *Desk Encyclopedia of Human and Medical Virology*, edited by B. W. J. Mahy & M. H. V. V. Regenmortel. Oxford: Elsevier-Academic Press. 249-256.
Clinical and Laboratory Standards Institute. 2013. *Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; approved standard VET01-A4*. 4th ed. Wayne, PA: Clinical and Laboratory Standards Institute (CLSI).
CLSI. 2008. *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline*. 3rd ed. Wayne, PA: Clinical and Laboratory Standards Institute (CLSI).
CO2Now. 2013. *Earth's CO2 Home Page*. CO2Now.org [accessed August 30, 2013. Available from http://co2now.org/Current-CO2/CO2-Now/.
Coffey, L.L., Page, B.L., Greninger, A.L., Herring, B.L., Russell, R.C., Doggett, S.L., Haniotis, J., Wang, C., Deng, X., & Delwart, E.L. 2014. Enhanced arbovirus surveillance with deep sequencing: Identification of novel rhabdoviruses and bunyaviruses in Australian mosquitoes. *Virology* 448:146-158.
Cogliati, M. 2013. Global molecular epidemiology of *Cryptococcus neoformans* and Cryptococcus gattii: an atlas of the molecular types. *Scientifica* 2013:23.
Cohen, J. 1988. *Statistical Power Analysis For The Behavioral Sciences*. 2nd ed. New Jersey: Lawrence Erlbaum Associates.

Cohn, J.P. 2008. White-nose syndrome threatens bats. *BioScience* 58 (11):1098.
Connolly, J.H., Canfield, P.J., Hemsley, S., & Spencer, A.J. 1998. Lymphoid neoplasia in the koala. *Australian Veterinary Journal* 76 (12):819-825.
Connolly, J.H., Krockenberger, M.B., Malik, R., Canfield, P.J., Wigney, D.I., & Muir, D.B. 1999. Asymptomatic carriage of *Cryptococcus neoformans* in the nasal cavity of the koala (*Phascolarctos cinereus*). *Medical Mycology* 37 (5):331-338.
Coombs, K.M. 2010. Reoviruses: Molecular Biology. In *Desk Encyclopedia of General Virology*, edited by B. W. J. Mahy & M. H. V. V. Regenmortel. Oxford, UK: Elsevier-Academic Press. 577-585.
Cowled, B., Ward, M.P., Hamilton, S., & Garner, G. 2009. The equine influenza epidemic in Australia: Spatial and temporal descriptive analyses of a large propagating epidemic. *Preventive Veterinary Medicine* 92 (1-2):60-70.
Cox, F.E., Wedderburn, N., & Salaman, M.H. 1974. The effect of Rowson-Parr virus on the severity of malaria in mice. *Journal of General Microbiology* 85 (2):358-364.
Cox, F.E.G. 2001. Concomitant infections, parasites and immune responses. *Parasitology* 122 (S1):S23-S38.
Crandall, L.S. 1964. *The Management of Wild Mammals in Captivity.* Chicago: Univesity of Chicago Press.
Croft, D.B. 1991. *Australian People And Animals In Today's Dreamtime: The Role Of Comparative Psychology In The Management Of Natural Resources*. Edited by E. Tobach, *Advances In Comparative Psychology Vol. 2*. New York: Praeger Publishers.
Crooks, K.R., Garcelon, D.K., Scott, C.A., Wilcox, J.T., Timm, S.F., & Van Vuren, D.H. 2003. Hematology and serum chemistry of the island spotted skunk on Santa Cruz Island. *Journal of Wildlife Diseases* 39 (2):460-466.
Cryan, P.M., Meteyer, C.U., Boyles, J.G., & Blehert, D.S. 2010. Wing pathology of white-nose syndrome in bats suggests life-threatening disruption of physiology. *BMC Biology* 8:135.
Cummings, K.J., Warnick, L.D., Alexander, K.A., Cripps, C.J., Gr√ ∂ hn, Y.T., James, K.L., McDonough, P.L., & Reed, K.E. 2009. The duration of fecal *Salmonella* shedding following clinical disease among dairy cattle in the northeastern USA. *Preventive Veterinary Medicine* 92 (1-2):134-139.
Cunningham, A.A., & Daszak, P. 1998. Extinction of a species of land snail due to infection with a microsporidian parasite. *Conservation Biology* 12 (5):1139-1141.
da Costa, M.M., Teixeira, F.M., Schalcher, T.R., de Brito, M.T.F.M., Valério, E.S., & Monteiro, M.C. 2013. Cryptococcosis, a risk for immunocompromised and immunocompetent individuals. *Open Epidemiology Journal* 6:9-17.
Dadachova, E., Bryan, R.A., Huang, X., Moadel, T., Schweitzer, A.D., Aisen, P., Nosanchuk, J.D., & Casadevall, A. 2007. Ionizing radiation changes the electronic properties of melanin and enhances the growth of melanized fungi. *PLoS One* 2 (5):e457.
Dallap Schaer, B.L., Aceto, H., Caruso, M.A., & Brace, M.A. 2012. Identification of predictors of salmonella shedding in adult horses presented for acute colic. *Journal of Veterinary Internal Medicine* 26 (5):1177-1185.
Danesi, P., Firacative, C., Cogliati, M., Otranto, D., Capelli, G., & Meyer, W. 2014. Multilocus sequence typing (MLST) and M13 PCR fingerprinting revealed heterogeneity amongst *Cryptococcus* species obtained from Italian veterinary isolates. *FEMS Yeast Research* 14 (6):897-909.
Daniels, C.W., & Belosevic, M. 1994. Serum antibody responses by male and female C57Bl/6 mice infected with Giardia muris. *Clinical and Experimental Immunology* 97 (3):424-429.
Daszak, P., & Cunningham, A.A. 1999. Extinction by infection. *Trends in Ecology & Evolution* 14 (7):279.
Daszak, P., Cunningham, A.A., & Hyatt, A.D. 2000. Emerging infectious diseases of wildlife-threats to biodiversity and human health. *Science* 287:443-449.

Davies, R. 2013. Salmonellosis. In *OIE Terrestrial Manual*: OIE Available from http://www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/.

Davison, A.J. 2002. Evolution of the herpesviruses. *Veterinary Microbiology* 86 (1-2):69-88.

Davison, A.J., Eberle, R., Ehlers, B., Hayward, G.S., McGeoch, D.J., Minson, A.C., Pellett, P.E., Roizman, B., Studdert, M.J., & Thiry, E. 2009. The order Herpesvirales. *Archives of Virology* 154 (1):171-177.

De Camps, S., Dubey, J.P., & Saville, W.J.A. 2008. Seroepidemiology of *Toxoplasma gondii* in zoo animals in selected zoos in the midwestern United States. *Journal of Parasitology* 94 (3):648-653.

de Oliveira, L.R., Moraes-Souza, H., Maltos, A.L., dos Santos, K.C., Molina, R.J., & Barata, C.H. 2013. *Aureobasidium pullulans* infection in a patient with chronic lymphocytic leukemia. *Revista da Sociedade Brasileira de Medicina Tropical* 46 (5):660-662.

de Oliveira, M.D.F.P., Vieira, M.D.G., Primo, J., Siqueira, I.C., Carvalho, E.M., Farr√©, L., Fatal, P.L., & Bittencourt, A.L. 2010. Flower cells in patients with infective dermatitis associated with HTLV-1. *Journal of Clinical Virology* 48 (4):288-290.

de Tores, P., Burbidge, A., Morris, K., & Friend, T. 2008. *Setonix brachyurus*. IUCN Red List of Threatened Species. Version 2015-3 [accessed 7th January, 2016. Available from http://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T20165A9156418.en.

de Tores, P.J., Hayward, M.W., Dillon, M.J., & Brazell, R.I. 2007. Review of the distribution, causes for the decline and recommendations for management of the quokka, *Setonix brachyurus* (Macropodidae: Marsupialia), an endemic macropodid marsupial from south-west Western Australia. *Conservation Science Western Australia* 6 (1):13-73.

Del Poeta, M., & Casadevall, A. 2012. Ten challenges on *Cryptococcus* and cryptococcosis. *Mycopathologia* 173 (5-6):303-310.

Department of Environment. 2013a. *Climate Change Impacts in Australia*. Australian Government [accessed 31st August, 2013. Available from http://www.environment.gov.au/climate-change/climate-science/impacts/wa.

Department of Environment. 2013b. *Setonix brachyurus: Species Profile and Threats Database*. Australian Government [accessed 4th July, 2013. Available from http://www.environment.gov.au/cgi-bin/sprat/public/publicspecies.pl?taxon_id=229 - population_information.

Department of Environment and Conservation. 2012a. Chuditch (*Dasyurus geoffroii*) Recovery Plan. Wildlife Management Program No. 54. Perth, WA. Department of Environment and Conservation. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.

Department of Environment and Conservation. 2012b. Western barred bandicoot (*Perameles bougainville bougainville*), Burrowing bettong (*Bettongia lesueur lesueur*) and banded hare-wallaby (*Lagostrophus fasciatus*) Recovery Plan. Wildlife Management Program No. 49. Perth. Department of Environment and Conservation. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.

Department of Environment and Conservation. 2013. Quokka (*Setonix brachyurus*) Recovery Plan. Wildlife Management Program No. 56. Perth, WA. Department of Environment and Conservation. [accessed 10th June, 2013]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.

Department of Health and Ageing. Australia. 2013. *National Notifiable Diseases Suveillance System*. Department of Health and Ageing [accessed September 12th 2013]. Available from http://www9.health.gov.au/cda/source/cda-index.cfm.

Department of Parks and Wildlife. 2014. Western Ringtail Possum (*Pseudocheirus occidentalis*) Recovery Plan. Wildlife Management Program No. 58. Perth.

Department of Parks and Wildlife. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.
Department of Parks and Wildlife. 2015. Numbat (*Myrmecobius fasciatus*) Recovery Plan. Wildlife Management Program No. 60. Perth. Department of Parks and Wildlife,. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.
Department of Parks and Wildlife (DPaW). 2007. *NatureMap: Mapping Western Australia's Biodiversity* [accessed 22th October, 2014. Available from http://naturemap.dpaw.wa.gov.au/.
Dhabhar, F.S., & McEwen, B.S. 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: A potential role for leukocyte trafficking. *Brain, Behavior, and Immunity* 11 (4):286-306.
Diallo, I., Carter, P.D., & Storie, G. 2013. Encephalomyocarditis virus infection in a splenectomised calf. *Australian Veterinary Journal* 91 (9):391-394.
Diamond, J.M. 1975. The island dilemma: Lessons of modern biogeographic studies for the design of natural reserves. *Biological Conservation* 7 (2):129-146.
Dickman, C.R. 1992. Conservation of Mammals in the Australasian Region: the Importance of Islands. In *Australia and the Global Environmental Crisis: Looking For Peaceful Solutions*, edited by J. N. C. a. J. M. Drew. Canberra: Academy Press. 175-214.
Dickson, J., & McNeice, R. 1982. Lipomatosis in a quokka (*Setonix brachyurus*). *Australian Veterinary Journal* 58 (5):218-218.
Dierenfeld, E.S., du Toit, R., & Miller, R.E. 1988. Vitamin E in captive and wild black rhinoceros (*Diceros bicornis*). *Journal of Wildlife Diseases* 24 (3):547-550.
Dobos-Kovacs, M., Meszaros, J., Pellerdy, L., & Balsai, A. 1974. Studies on source of Toxoplasma infection in captive kangaroos. *Acta Veterinaria Academiae Scientiarum Hungaricae* 24 (3):293-301.
Doherty, R.L. 1972. Arboviruses of Australia. *Australian Veterinary Journal* 48 (4):172-180.
Donehower, L.A., Bohannon, R.C., Ford, R.J., & Gibbs, R.A. 1990. The use of primers from highly conserved pol regions to identify uncharacterized retroviruses by the polymerase chain reaction. *Journal of Virological Methods* 28 (1):33-46.
Donham, K.J., & Leininger, J.R. 1977. Spontaneous leprosy like disease in a chimpanzee. *Journal of Infectious Diseases* 136 (1):132-136.
Dubey, J.P. 2004. Toxoplasmosis - a waterborne zoonosis. *Veterinary Parasitology* 126 (1-2):57-72.
Dubey, J.P., & Crutchley, C. 2008. Toxoplasmosis in wallabies (*Macropus rufogriseus* and *Macropus eugenii*): Blindness, treatment with atovaquone, and isolation of *Toxoplasma gondii*. *Journal of Parasitology* 94 (4):929-933.
Dubey, J.P., & Odening, K. 2001. Toxoplasmosis and Related Infections. In *Parasitic Diseases of Wild Mammals*, edited by W. M. Samuel, M. J. Pybus & A. A. Kocan. 2nd ed. Ames, IA: Iowa State University Press. 478-519.
Duffy, L.L., Dykes, G.A., & Fegan, N. 2012. A review of the ecology, colonization and genetic characterization of *Salmonella enterica* serovar Sofia, a prolific but avirulent poultry serovar in Australia. *Food Research International* 45 (2):770-779.
Duncan, C., Schwantje, H., Stephen, C., Campbell, J., & Bartlett, K. 2006. *Cryptococcus gattii* in wildlife of Vancouver Island, British Columbia, Canada. *Journal of Wildlife Diseases* 42 (1):175-178.
Dunnet, G.M. 1962. A population study of the quokka, *Setonix brachyurus* (Quoy & Gaimar) (Marsupialia) II. Habitat, movements, breeding, and growth. *Wildlife Research* 7:13-32.
Dunnet, G.M. 1963. A population study of the quokka, *Setonix brachyurus* Quoy & Gaimard (Marsupialia) III. The estimation of population parameters by means of the recapture technique. *Wildlife Research* 8:78-117.
Durham, P.J., Finnie, J.W., Lawrence, D.A., & Alexander, P. 1996. Blindness in South Australian kangaroos. *Australian Veterinary Journal* 73 (3):111-112.

Duszynski, D.W., & Upton, S.J. 2001. Cyclospora, Eimeria, Isospora and Cryptosporidium spp. In *Parasitic Diseases of Wild Mammals*, edited by W. M. Samuel, M. J. Pybus & A. A. Kocan. 2nd ed. Ames, IA: Iowa State University Press. 416-459.

Dwinger, R.H., Agyemang, K., Kaufmann, J., Grieve, A.S., & Bah, M.L. 1994. Effects of trypanosome and helminth infections on health and production parameters of village N'Dama cattle in The Gambia. *Veterinary Parasitology* 54 (4):353-365.

Edgar, R.C. 2004. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32 (5):1792-1797.

Effron, M., Griner, L., & Benirschke, K. 1977. Nature and rate of neoplasia found in captive wild mammals, birds, and reptiles at necropsy. *Journal of the National Cancer Institute* 59 (1):185-198.

Efron, B., Halloran, E., & Holmes, S. 1996. Bootstrap confidence levels for phylogenetic trees. *Proceedings of the National Academy of Sciences of the United States of America* 93 (14):7085-7090.

Erickson, R. 1951. Quokka feeding on introduced snail and stinkwort. *The Western Australian Naturalist* 3:41.

Eriksson, E., & Aspan, A. 2007. Comparison of culture, ELISA and PCR techniques for salmonella detection in faecal samples for cattle, pig and poultry. *BMC Veterinary Research* 3:21.

Eymann, J., Herbert, C.A., Cooper, D.W., & Dubey, J.P. 2006. Serologic survey for Toxoplasma gondii and Neospora caninum in the common brushtail possum (Trichosurus vulpecula) from urban Sydney, Australia. *Journal of Parasitology* 92 (2):267-272.

Farrell, P.M., Bieri, J.G., Fratantoni, J.F., Wood, R.E., & di Sant'Agnese, P.A. 1977. The occurrence and effects of human vitamin E deficiency. A study in patients with cystic fibrosis. *Journal of Clinical Investigation* 60 (1):233-241.

Feore, S.M., Bennett, M., Chantrey, J., Jones, T., Baxby, D., & Begon, M. 1997. The effect of cowpox virus infection on fecundity in bank voles and wood mice. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 264 (1387):1457-1461.

Festa-Bianchet, M. 1989. Individual differences, parasites, and the costs of reproduction for bighorn ewes (*Ovis canadensis*). *Journal of Animal Ecology* 58 (3):785-795.

Finnie, E.P., Littlejohns, I.R., & Acland, H.M. 1976. Mortalities in parma wallabies (*Macropus parma*) associated with probable herpesvirus. *Australian Veterinary Journal* 52 (6):294-294.

Fleming, P.A., Harman, A.M., & Beazley, L.D. 1996. Development and ageing of the RPE in a marsupial, the quokka. *Experimental Eye Research* 62 (5):457-469.

Flynn, J.J., Finarelli, J.A., Zehr, S., Hsu, J., & Nedbal, M.A. 2005. Molecular phylogeny of the Carnivora (Mammalia): assessing the impact of increased sampling on resolving enigmatic relationships. *Systematic Biology* 54 (2):317-337.

Fothergill, A.W. 2007. Medically significant fungi. In *Diagnostic Microbiology*, edited by C. R. Mahon, D. C. Lehman & G. Manuselis. 3rd ed. St. Louis: Saunders ELSEVIER. 718-760.

Fowler, A., & Mitchell, T. 2002. Skin lesions in a free-ranging population of long-footed potoroos (*Potorous longipes*) in east Gippsland. Paper read at *Wildlife Disease Association (Australian Section) Annual Conference*, at Mary River, Australia.

Fox, J.G., Beaucage, C.M., Murphy, J.C., & Niemi, S.M. 1984. Experimental Salmonella-associated conjunctivitis in cats. *Canadian Journal of Comparative Medicine* 48 (1):87-91.

Freegard, C., & Richter, V. 2009a. Standard Operating Procedure No. 9.2: Cage traps for live capture of terrestrial invertebrates. Perth, WA: Department of Parks and Wildlife. Government of Western Australia. [accessed 13th June, 2012]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/96-monitoring/standards/99-standard-operating-procedures.

Freegard, C., & Richter, V. 2009b. Standard Operating Procedure No. 9.5: Soft cage traps for capture of macropods. Perth, WA: Department of Parks and Wildlife. Government of Western Australia. [accessed 13th June, 2012]. Available from

http://www.dpaw.wa.gov.au/plants-and-animals/96-monitoring/standards/99-standard-operating-procedures.
Freegard, C., & Richter, V. 2009c. Standard Operating Procedure No. 14.1: Care of evicted pouch young. Perth, WA: Department of Parks and Wildlife. Government of Western Australia. [accessed 13th June, 2012]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/96-monitoring/standards/99-standard-operating-procedures.
Friedman, E.M., & Lawrence, D.A. 2002. Environmental stress mediates changes in neuroimmunological interactions. *Toxicological Sciences* 67 (1):4-10.
Fukuda, R.I., Hayashi, A., Utsunomiya, A., Nukada, Y., Fukui, R., Itoh, K., Tezuka, K., Ohashi, K., Mizuno, K., Sakamoto, M., Hamanoue, M., & Tsuji, T. 2005. Alteration of phosphatidylinositol 3-kinase cascade in the multilobulated nuclear formation of adult T cell leukemia/lymphoma (ATLL). *Proceedings of the National Academy of Sciences of the United States of America* 102 (42):15213-15218.
Gabor, C.R., Fisher, M.C., & Bosch, J. 2013. A non-invasive stress assay shows that tadpole populations infected with *Batrachochytrium dendrobatidis* have elevated corticosterone levels. *PLoS One* 8 (2):e56054.
Gagnon, C.A., Tremblay, J., Larochelle, D., Music, N., & Tremblay, D. 2011. Identification of a novel herpesvirus associated with cutaneous ulcers in a fisher (*Martes pennanti*). *Journal of Veterinary Diagnostic Investigation* 23 (5):986-990.
Gainer, J.H., & Bigler, W.I. 1967. Encephalomyocarditis (EMC) virus recovered from two cotton rats and a raccoon. *Journal of Wildlife Diseases* 3 (2):47-49.
Gales, N., Wallace, G., & Dickson, J. 1985. Pulmonary ryptococcosis in a striped dolphin (*Stenella coeruleoalba*). *Journal of Wildlife Diseases* 21 (4):443-446.
Gamble, K.C. 2004. Marsupial care and husbandry. *Veterinary Clinics of North America - Exotic Animal Practice* 7 (2):283-298.
Garcia, M.J., & Gola, S. 2016. Gene and whole genome analyses reveal that the mycobacterial strain JS623 is not a member of the species *Mycobacterium smegmatis*. *Microbial Biotechnology* 9 (2):269-274.
Garcia-Hermoso, D., Janbon, G., & Dromer, F. 1999. Epidemiological evidence for dormant Cryptococcus neoformans infection. *Journal of Clinical Microbiology* 37 (10):3204-3209.
Garver, K.A., Ai-Hussinee, L., Hawley, L.M., Schroeder, T., Edes, S., LePage, V., Contador, E., Russell, S., Lord, S., Stevenson, R.M.W., Souter, B., Wright, E., & Lumsden, J.S. 2010. Mass mortality associated with koi herpesvirus in wild common carp in Canada. *Journal of Wildlife Diseases* 46 (4):1242-1251.
Gasner, M.R., Jankowski, J.E., Ciecka, A.L., Kyle, K.O., & Rabenold, K.N. 2010. Projecting the local impacts of climate change on a Central American montane avian community. *Biological Conservation* 143 (5):1250-1258.
Geffre, A., Concordet, D., Braun, J.P., & Trumel, C. 2011. Reference Value Advisor: a new freeware set of macroinstructions to calculate reference intervals with Microsoft Excel. *Veterinary Clinical Pathology* 40 (1):107-112.
Geffré, A., Concordet, D., Trumel, C., & Braun, J.P. 2011. Validation of preexisting reference intervals: Can the procedure be applied to canine hemostasis? *Journal of Veterinary Diagnostic Investigation* 23 (2):343-347.
Gibb, D.G.A., Kakulas, B.A., Perret, D.H., & Jenkyn, D.J. 1966. Toxoplasmosis in the Rottnest quoka (*Setonix brachyurus)*. *Australian Journal of Experimental Biology and Medical Science* 44:665-672.
Gibbs, A. 1987. Molecular evolution of viruses; 'trees', 'clocks' and 'modules'. *Journal of Cell Science. Supplement* 7 (Journal Article):319.
Gibson, L., McNeill, A., Tores, P.D., Wayne, A., & Yates, C. 2010. Will future climate change threaten a range restricted endemic species, the quokka (*Setonix brachyurus*), in south west Australia? *Biological Conservation* 143 (11):2453-2461.
Glenister, B.F., Hassell, C.W., & Kneebone, E.W.S. 1959. Geology of Rottnest Island. *Journal of the Royal Society of Western Australia* 42:69 -70.

Gog, J., Woodroffe, R., & Swinton, J. 2002. Disease in endangered metapopulations: The importance of alternative hosts. *Proceedings: Biological Sciences* 269 (1492):671-676.

Gole, C. 2006. The Southwest Australia Ecoregion: Jewel of the Australian Continent. Wembley. WWF-Australia. [accessed August 28, 2013]. Available from http://wwf.org.au/our_work/saving_the_natural_world/australian_priority_places/southwest_australia/southwest_australia_ecoregion/.

Gould, J. 1863. *The Mammals of Australia*. Vol. 1. London: 26 Charlotte Street, Bedford Square.

Government of Western Australia. 2010. *Heritage icons: May - Rottnest Island*. The Constitutional Centre of Western Australia [accessed 9th July, 2012. Available from http://www.ccentre.wa.gov.au/175thAnniversary/HeritageIcons/Pages/May-RottnestIsland.aspx.

Granados, D.P., & Castañeda, E. 2005. Isolation and characterization of *Cryptococcus neoformans* varieties recovered from natural sources in Bogotá, Colombia, and study of ecological conditions in the area. *Microbial Ecology* 49 (2):282-290.

Green, J.R., & Bulmer, G.S. 1979. Gastrointestinal inoculation of *Cryptococcus neoformans* in mice. *Sabouraudia* 17 (3):233-240.

Grimont, P.A.D., & Weill, F.-X. 2007. *Antigenic formulae of the Salmonella serovars*. 9th Edition ed. Paris: Institute Pasteur, World Health Organization Collaborating Center for Reference and Research on Salmonella.

Griner, L.A. 1977. Cryptococcosis (Torulosis, European blastomycosis) in zoo animals. In *Internationalen Symposiums uber die Erkrankungen der Zootiere XIX*, 243-249.

Grobler, D.G., Raath, J.P., Braack, L.E., Keet, D.F., Gerdes, G.H., Barnard, B.J., Kriek, N.P., Jardine, J., & Swanepoel, R. 1995. An outbreak of encephalomyocarditis-virus infection in free-ranging African elephants in the Kruger National Park. *Onderstepoort Journal of Veterinary Research* 62 (2):97-108.

Gubser, C., Hue, S., Kellam, P., & Smith, G.L. 2004. Poxvirus genomes: a phylogenetic analysis. *Journal of General Virology* 85 (Pt 1):105-117.

Guiler, E.R. 1961. The former distribution and decline of the thylacine. *Australian Journal of Science* 23 (7):207-210.

Guliani, S., Smith, G.A., Young, P.L., Mattick, J.S., & Mahony, T.J. 1999. Reactivation of a macropodid herpesvirus from the eastern grey kangaroo (*Macropus giganteus*) following corticosteroid treatment. *Veterinary Microbiology* 68 (1-2):59-69.

Gyuranecz, M., Foster, J.T., Dán, Á., Ip, H.S., Egstad, K.F., Parker, P.G., Higashiguchi, J.M., Skinner, M.A., Höfle, U., Kreizinger, Z., Dorrestein, G.M., Solt, S., Sós, E., Kim, Y.J., Uhart, M., Pereda, A., González-Hein, G., Hidalgo, H., Blanco, J.-M., & Erdélyi, K. 2013. Worldwide phylogenetic relationship of avian poxviruses. *Journal of Virology* 87 (9):4938-4951.

Hadas, E., Derda, M., Winiecka-Krusnell, J., & Sutek, A. 2004. Acanthamoeba spp. as vehicles of pathogenic bacteria. *Acta Parasitologica* 49 (4):276-280.

Haley, B.J., Cole, D.J., & Lipp, E.K. 2009. Distribution, diversity, and seasonality of waterborne salmonellae in a rural watershed. *Applied and Environmental Microbiology* 75 (5):1248-1255.

Hall, M.L.M., & Rowe, B. 1992. Salmonella arizonae in the United Kingdom from 1966 to 1990. *Epidemiology and Infection* 108 (1):59-65.

Hamilton, P.B., Stevens, J.R., Gidley, J., Holz, P., & Gibson, W.C. 2005. A new lineage of trypanosomes from Australian vertebrates and terrestrial bloodsucking leeches (Haemadipsidae). *International Journal for Parasitology* 35 (4):431-443.

Hammer, Ø., Harper, D.A.T., & Ryan, P.D. 2001. Past: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* 4 (1):XIX-XX.

Hanssen, S.A., Hasselquist, D., Folstad, I., & Erikstad, K.E. 2004. Costs of immunity: immune responsiveness reduces survival in a vertebrate. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 271 (1542):925-930.

Hart, R.P. 1980. Ecology of the salmonella in a wild marsupial, the quokka *Setonix brachyurus*. Ph.D. diss., Zoology, University of Western Australia, Perth.
Hart, R.P., Bradshaw, S.D., & Iveson, J.B. 1985. *Salmonella* infections in a marsupial, the quokka (*Setonix brachyurus*), in relation to seasonal changes in condition and environmental stress. *Applied and Environmental Microbiology* 49 (5):1276-1281.
Hart, R.P., Bradshaw, S.D., & Iveson, J.B. 1986. Salmonella infections and animal condition in the mainland and Bald Island populations of the quokka (*Setonix brachyurus*: Marsupialia). *Journal of the Royal Society of Western Australia* 69 (1):7-11.
Hart, R.P., Iveson, J.B., & Bradshaw, S.D. 1987. The ecology of *Salmonella* serotypes in a wild marsupial (the quokka *Setonix brachyurus*) in a disturbed environment. *Australian Journal of Ecology* 12:267-279.
Hart, R.P., Iveson, J.B., Bradshaw, S.D., & Speed, T.P. 1982. A study of isolation procedures for multiple infections of *Salmonella* and *Arizona* in a wild marsupial, the quokka (*Setonix brachyurus*). *Journal of Applied Bacteriology* 53:395-406.
Hartigan, A., Fiala, I., Dykova, I., Jirku, M., Okimoto, B., Rose, K., Phalen, D.N., & Slapeta, J. 2011. A suspected parasite spill-back of two novel *Myxidium* spp. (Myxosporea) causing disease in Australian endemic frogs found in theinvasive cane toad. *PLoS One* 6 (4):e18871.
Hartley, M., & English, A. 2005. A seroprevalence survey of Toxoplasma gondii in common wombats (*Vombatus ursinus*). *European Journal of Wildlife Research* 51 (1):65-67.
Hartley, W.J. 1993. Central nervous system disorders in the brush-tail possum in Eastern Australia. *New Zealand Veterinary Journal* 41 (1):44-45.
Hartnack, A.K., van Metre, D.C., & Morley, P.S. 2012. Salmonella enterica shedding in hospitalized horses and associations with diarrhea occurrence among their stablemates and gastrointestinal-related illness or death following discharge. *Journal of the American Veterinary Medical Association* 240 (6):726-733.
Harvell, C.D., Mitchell, C.E., Ward, J.R., Altizer, S., Dobson, A.P., Ostfeld, R.S., & Samuel, M.D. 2002. Climate warming and disease risks for terrestrial and marine biota. *Science* 296 (5576):2158-2162.
Harvey, J.W. 2012. *Veterinary Hematology: A Diagnostic Guide and Color Atlas*. St. Louis, USA: ELSEVIER SAUNDERS.
Hawkey, C.M. 1975. Other mammalian orders. In *Comparative Mammalian Haematology: Cellular Components and Blood Coagulation of Captive WIld Animals*. London: William Heinemann Medical Books. 218-273.
Hawkins, C.E., Baars, C., Hesterman, H., Hocking, G.J., Jones, M.E., Lazenby, B., Mann, D., Mooney, N., Pemberton, D., Pyecroft, S., Restani, M., & Wiersma, J. 2006. Emerging disease and population decline of an island endemic, the Tasmanian devil *Sarcophilus harrisii*. *Biological Conservation* 131 (2):307-324.
Hayward, M. 2002. The ecology of the quokka (*Setonix brachyurus*) (Macropodidae: Marsupialia) in the Northern Jarrah Forest of Australia. Ph.D. diss., School of Biological, Earth and Environmental Science, University of New South Wales, Perth.
Hayward, M.W. 2005. Diet of the quokka (*Setonix brachyurus*) (Macropodidae:Marsupialia) in the northern jarrah forest of Western Australia. *Wildlife Research* 32 (1):15-22.
Hayward, M.W. 2008. Home range overlap of the quokka *Setonix brachyurus* (Macropodidae: Marsupialia) suggets a polygynous mating system. *Conservation Science Western Australia* 7 (1):57-64.
Hayward, M.W., de Tores, P.J., Augee, M.L., Fox, B.J., & Banks, P.B. 2004. Home range and movements of the quokka, *Setonix brachyurus* (Macropodidae:Marsupialia), and its impact on the viability of the metapopulation on the Australian mainland. *Journal of Zoology* 263 (3):219-228.
Hayward, M.W., de Tores, P.J., Dillon, M.J., & Banks, P.B. 2007. Predicting the ocurrence of the quokka, *Setonix brachyurus* (Macropodidae:Marsupialia), in Western Australia's northern jarrah forest. *Wildlife Research* 34 (3):194-199.

Hayward, M.W., De Tores, P.J., Dillon, M.J., & Fox, B.J. 2003. Local population structure of a naturally occurring metapopulation of the quokka (*Setonix brachyurus* Macropodidae: Marsupialia). *Biological Conservation* 110 (3):343-355.
Heard, M.J., Smith, K.F., Ripp, K.J., Berger, M., Chen, J., Dittmeier, J., Goter, M., McGarvey, S.T., & Ryan, E. 2013. The threat of disease increases as species move toward extinction. *Conservation Biology*.
Heckscher, S.K., Wickesberg, B.A., Duszynski, D.W., & Gardner, S.L. 1999. Three new species of Eimeria from Bolivian marsupials. *International Journal for Parasitology* 29 (2):275-284.
Hess, G.R. 1994. Conservation corridors and contagious disease: A cautionary note. *Conservation Biology* 8 (1):256-262.
Hilbert, F., Smulders, F.J.M., Chopra-Dewasthaly, R., & Paulsen, P. 2012. Salmonella in the wildlife-human interface. *Food Research International* 45 (2):603-608.
Hill, B.M., & Ward, S.J. 2010. National Recovery Plan for the Northern Quoll (*Dasyurus hallucatus*). Darwin. Department of Natural Resources, Environment, The Arts and Sport. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.
Hill, D.E., Chirukandoth, S., Dubey, J.P., Lunney, J.K., & Gamble, H.R. 2006. Comparison of detection methods for *Toxoplasma gondii* in naturally and experimentally infected swine. *Veterinary Parasitology* 141 (1-2):9-17.
Hill, N.J., Richter, C., & Power, M.L. 2012. Pinning down a polymorphic parasite: New genetic and morphological descriptions of *Eimeria macropodis* from the Tammar wallaby (*Macropus eugenii*). *Parasitology International* 61 (3):461-465.
Hing, S., Narayan, E.J., Thompson, R.A., & Godfrey, S.S. 2016. The relationship between physiological stress and wildlife disease: consequences for health and conservation. *Wildlife Research* 43 (1):51-60.
Hirsh, D.C. 2004. Enterobacteriaceae: Salmonella. In *Veterinary Microbiology*, edited by D. C. Hirsh, N. J. MacLachlan & R. L. Walker. 2nd ed. Ames: Blackwell Publishing. 69-74.
Hoang, L.M.N., Maguire, J.A., Doyle, P., Fyfe, M., & Roscoe, D.L. 2004. *Cryptococcus neoformans* infections at Vancouver Hospital and Health Sciences Centre (1997-2002): epidemiology, microbiology and histopathology. *Journal of Medical Microbiology* 53 (9):935-940.
Hodgkin, E.P. 1959. The salt lakes of Rottnest Island. *Journal of the Royal Society of Western Australia* 42:84-85.
Hofer, H., & East, M. 2012. Stress and immunosuppression as factors in the decline and extinction of wildlife populations: concepts, evidence and challenges. In *New Directions In Conservation Medicine: Applied Cases Of Ecological Health*, edited by A. A. Aguirre, R. S. Ostfeld & P. Daszak. New York: Oxford University Press. 82-107.
Holsworth, W.N. 1964. Marsupial behaviour : with special reference to population homeostasis in the quokkas on the west end of Rottnest island. Ph.D. dissertation, Faculty of Science, University of Western Australia, Perth.
Hooper, P.T., Lunt, R.A., Gould, A.R., Hyatt, A.D., Russell, G.M., Kattenbelt, J.A., Blacksell, S.D., Reddacliff, L.A., Kirkland, P.D., Davis, R.J., Durham, P.J.K., Bishop, A.L., & Waddington, J. 1999. Epidemic of blindness in kangaroos - evidence of a viral aetiology. *Australian Veterinary Journal* 77 (8):529-536.
Hsu, M.C., Chen, K.W., Lo, H.J., Chen, Y.C., Liao, M.H., Lin, Y.H., & Li, S.Y. 2003. Species identification of medically important fungi by use of real-time LightCycler PCR. *Journal of Medical Microbiology* 52 (12):1071-1076.
Huang, C.J., & Fwu, M.L. 1993. Degree of protein deficiency affects the extent of the depression of the antioxidative enzyme activities and the enhancement of tissue lipid peroxidation in rats. *The Journal of nutrition* 123 (5):803.
Hubálek, Z., Rudolf, I., & Nowotny, N. 2014. Arboviruses pathogenic for domestic and wild animals. *Advances in Virus Research* 89:201-275.

Hubbard, G.B., Schmidt, R.E., & Fletcher, K.C. 1983. Neoplasia in Zoo Animals. *Journal of Zoo Animal Medicine* 14 (1):33-40.
Huff, J.L., & Barry, P.A. 2003. B-virus (Cercopithecine herpesvirus 1) infection in humans and macaques: potential for zoonotic disease. *Emerging Infectious Diseases* 9 (2):246-250.
Hume, I.D., Jarman, P.J., Renfree, M.B., & Temple-Smith, P.D. 1989. Macropodidae. In *Fauna Of Australia*, edited by D. W. Walton & B. J. Richardson. Canberra: Australian Government Publishing Service. 679-715.
Hüssy, D., Janett, F., Albini, S., Stäuber, N., Thun, R., & Ackermann, M. 2002. Analysis of the pathogenetic basis for shedding and transmission of ovine gamma herpesvirus 2. *Journal of Clinical Microbiology* 40 (12):4700-4704.
Huttova, M., Kralinsky, K., Horn, J., Marinova, I., Iligova, K., Fric, J., Spanik, S., Filka, J., Uher, J., Kurak, J., & Krcmery Jr, V. 1998. Prospective study of nosocomial fungal meningitis in children - Report of 10 cases. *Scandinavian Journal of Infectious Diseases* 30 (5):485-487.
Illnait-Zaragozi, M.T., Hagen, F., Fernandez-Andreu, C.M., Martinez-Machin, G.F., Polo-Leal, J.L., Boekhout, T., Klaassen, C.H., & Meis, J.F. 2011. Reactivation of a *Cryptococcus gattii* infection in a cheetah (*Acinonyx jubatus*) held in the National Zoo, Havana, Cuba. *Mycoses* 54 (6):e889-892.
Ilmonen, P., Taarna, T., & Hasselquist, D. 2000. Experimentally activated immune defence in female pied flycatchers results in reduced breeding success. *Proceedings of the Royal Society of London, Series B: Biological Sciences* 267 (1444):665-670.
Ishihara, K., Kitagawa, H., Yokoyama, S., & Ohashi, H. 1981. Studies on hemolysis in canine dirofilarial hemoglobinuria. Lipid alterations in blood serum and red cell membrane. *Nippon Juigaku Zasshi. The Japanese Journal of Veterinary Science* 43 (1):1-11.
IUCN. 2015. *The IUCN Red List of Threatened Species. Version 2015-4* [accessed 13 December 2015]. Available from http://www.iucnredlist.org.
Iveson, J.B. 1977. *Salmonella* infections in wildlife in Western Australia, a natural barometer of environmental health. In *Australian Institute of Health Surveyors, 4th National Conference*, *Perth*, *October 1977*.
Iveson, J.B., & Bradshaw, S.D. 1973. *Salmonella* javiana Infection in an infant associated with a marsupial, the quokka, *Setonix brachyurus*, in Western Australia. *Journal of Hygiene* 71 (3):423-432.
Iveson, J.B., Bradshaw, S.D., & Hart, R.P. 2007. Seasonal and spatial variation in *Salmonella* infections rates in quokkas (*Setonix brachyurus*) on Rottnest Island, Western Australia, in areas of human contact. *Journal of the Royal Society of Western Australia* 90:127-136.
Iveson, J.B., & Hart, R.P. 1983. *Salmonella* on Rottnest Island: Implications for pubic health and wildlife management. *Journal of the Royal Society of Western Australia* 66 (Parts 1 and 2):15-20.
Jackson, R., Cooke, M.M., Coleman, J.D., & Morris, R.S. 1995. Naturally occurring tuberculosis caused by *Mycobacterium bovis* in brushtail possums (*Trichosurus vulpecula*): I. An epidemiological analysis of lesion distribution. *New Zealand Veterinary Journal* 43 (7):306-314.
Jacqueline, D.R. 2012. Rufous hare-wallaby (*Lagorchestes hirsutus*) National Recovery Plan. Australian Wildlife Management Program No. 43. Perth, WA. Department of Parks and Wildlife. [accessed 17th January, 2013]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.
Jacups, S.P., Whelan, P.I., & Currie, B.J. 2008. Ross River virus and Barmah Forest virus infections: A review of history, ecology, and predictive models, with implications for tropical northern Australia. *Vector-Borne and Zoonotic Diseases* 8 (2):283-297.
Jain, P., & Prabhash, K. 2010. Flower cells of leukemia. *Blood* 115 (9):1668.
Janbon, G., Ormerod, K.L., Paulet, D., Byrnes III, E.J., Yadav, V., Chatterjee, G., Mullapudi, N., Hon, C.-C., Billmyre, R.B., & Brunel, F. 2014. Analysis of the genome and

transcriptome of *Cryptococcus neoformans* var. *grubii* reveals complex rna expression and microevolution leading to virulence attenuation. *PLoS Genetics* 10 (4):e1004261.
Jarmin, S., Manvell, R., Gough, R.E., Laidlaw, S.M., & Skinner, M.A. 2006. Avipoxvirus phylogenetics: identification of a PCR length polymorphism that discriminates between the two major clades. *Journal of General Virology* 87 (8):2191-2201.
Jefferies, R., Ryan, U.M., & Irwin, P.J. 2007. PCR-RFLP for the detection and differentiation of the canine piroplasm species and its use with filter paper-based technologies. *Veterinary Parasitology* 144 (1-2):20-27.
Jeffery, J.A.L., Ryan, P.A., Lyons, S.A., & Kay, B.H. 2002. Vector competence of *Coquillettidia linealis* (Skuse) (Diptera: Culicidae) for Ross River and Barmah Forest viruses. *Australian Journal of Entomology* 41 (4):339-344.
Johansen, C.A., Mackenzie, J.S., Smith, D.W., & Lindsay, M.D.A. 2005. Prevalence of neutralising antibodies to Barmah Forest, Sindbis and Trubanaman viruses in animals and humans in the south-west of Western Australia. *Australian Journal of Zoology* 53 (1):51-58.
Johnson, A.J., Pessier, A.P., Wellehan, J.F.X., Brown, R., & Jacobson, E.R. 2005. Identification of a novel herpesvirus from a California desert tortoise (*Gopherus agassizii*). *Veterinary Microbiology* 111 (1-2):107-116.
Johnson, A.M., Roberts, H., & Munday, B.L. 1988. Prevalence of *Toxoplasma gondii* antibody in wild macropods. *Australian Veterinary Journal* 65 (7):199-201.
Johnson, A.M., Roberts, H., Statham, P., & Munday, B.L. 1989. Serodiagnosis of acute toxoplasmosis in macropods. *Veterinary Parasitology* 34 (1-2):25-33.
Johnson, C. 2006. *Australia's mammal extinctions: a 50,000-year history*. New York: Cambridge University Press.
Johnson, C.N., & Isaac, L.J. 2009. Body mass and extinction risk in Australian marsupials: The 'Critical Weight Range' revisited. *Austral Ecology* 34:35-40.
Johnson, K.A., Burbidge, A.A., & McKenzie, N.L. 1989. Australian Macropodoidea: status, causes of decline and future research and management. In *Kangaroos, Wallabies and Rat Kangaroos*, edited by G. Grigg, P. Jarman & I. Hume. Surrey Beatty: N.S.W.: Surrey Beatty & Sons Pty. Limited. 641-657.
Johnson, L.L., & Boyce, M.S. 1991. Female choice of males with low parasite loads in sage grouse. In *Bird-parasite Interactions: Ecology, Evolution, and Behaviour*, edited by J. E. Loye & M. Zuk. Oxford: Oxford University Press. 377-388.
Johnson, M.A., & Whalley, J.M. 1987. Restriction enzyme maps of the macropodid herpesvirus 2 genome. *Archives of Virology* 96 (3-4):153-168.
Johnson, M.A., & Whalley, J.M. 1990. Structure and physical map of the genome of parma wallaby herpesvirus. *Virus Research* 18 (1):41-48.
Johnson, M.A., Whalley, J.M., Littlejohns, I.R., Dickson, J., Smith, V.W., Wilks, C.R., & Reisner, A.H. 1985. Macropodid herpesviruses 1 and 2: Two herpesviruses from Australian marsupials differentiated by restriction endonucleases, DNA composition and hybridization. *Archives of Virology* 85:313-319.
Johnson, R., & Hemsley, S. 2010. Glidders and Possums. In *Medicine of Australian Mammals*, edited by L. Vogelnest & R. Woods. Collingwood, Victoria: CSIRO Publishing. 395-438.
Jones, F.W. 1924. *The Mammals of South Australia. The Bandicoots and Herbivorous Marsupials*. Vol. 2. Adelaide: Govt. Printer.
Jones, J.B., Hyatt, A.D., Hine, P.M., Whittington, R.J., Griffin, D.A., & Bax, N.J. 1997. Australasian pilchard mortalities. *World Journal of Microbiology and Biotechnology* 13 (4):383-392.
Jordan, S.M., & Morgan, E.H. 1968. The changes in the serum and milk whey proteins during lactation and suckling in the quokka (*Setonix brachyurus*). *Comparative Biochemistry and Physiology* 25 (1):271-283.
Kadota, K., Uchida, K., Nagatomo, T., Goto, Y., Shinjo, T., Hasegawa, T., Ogawa, H., Yamaguchi, R., & Tateyama, S. 1995. Granulomatous epididymitis related to *Rhodotorula glutinis* infection in a dog. *Veterinary Pathology* 32 (6):716-718.

Kakulas, B.A. 1961. Myopathy affecting the Rottnest Quokka (*Setonix brachyurus*) reversed by alpha-Tocopherol. *Nature* 191 (4786):402-403.
Kakulas, B.A. 1963a. Influence of the size of enclosure on the development of myopathy in the captive Rottnest quokka. *Nature* 198 (4881):673-674.
Kakulas, B.A. 1963b. Trace quantities of selenium ineffective in the prevention of nutritional myopathy in the Rottnest quokka (*Setonix brachyurus*). *Australian Journal of Science* 25:313-314.
Kakulas, B.A. 1964. Atypical acid-fast bacillary inection in the Rottnest quokka (*Setonix brachyurus*). *Australian Journal of Science* 27 (4):115-116.
Kakulas, B.A. 1983. The nutritional myopathy of the quokka as a model for research in muscular dystrophy. *Journal of the Royal Society of Western Australia* 66:52-55.
Kakulas, B.A., & Adams, R.D. 1966. Principles of myopathology as illustrated in the nutritional myopathy of the Rottnest quokka (*Setonix brachyurus*). *Annals of the New York Academy of Sciences* 138 (1):90-101.
Kaldor, I., & Morgan, E.H. 1986. Iron metabolism during lactation and suckling in a marsupial, the quokka (*Setonix brachyurus*). *Comparative Biochemistry and Physiology Part A: Physiology* 84 (4):691-694.
Kapikian, A.Z., & Shope, R.E. 1996. Rotaviruses, Reoviruses, Coltiviruses, and Orbiviruses. In *Medical Microbiology*, edited by S. Baron. 4th ed. Galveston, TX: University of Texas Medical Branch.
Kärber, G. 1931. Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Naunyn-Schmiedebergs Archiv fär Experimentelle Pathologie und Pharmakologie* 162 (4):480-483.
Katsu, M., Kidd, S., Ando, A., Moretti-Branchini, M.L., Mikami, Y., Nishimura, K., & Meyer, W. 2004. The internal transcribed spacers and 5.8S rRNA gene show extensive diversity among isolates of the *Cryptococcus neoformans* species complex. *FEMS Yeast Research* 4 (4-5):377-388.
Kay, B.H., Hall, R.A., Fanning, I.D., Mottram, P., Young, P.L., & Pollitt, C.C. 1986. Experimental infection of vertebrates with Murray Valley Encephalitis and Ross River Viruses. In *Arbovirus Research In Australia, 4th Symposium Proceedings*, *Brisbane*, *May 6-9, 1986*, 71-75.
Kerr, P.J., & Best, S.M. 1998. Myxoma virus in rabbits. *OIE Revue Scientifique et Technique* 17 (1):256-268.
Khan, A., Dubey, J.P., Su, C., Ajioka, J.W., Rosenthal, B.M., & Sibley, L.D. 2011. Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. *International Journal for Parasitology* 41 (6):645-655.
Khan, A.B., Sahir, K.H., Ahmed, M., & Khan, S.I. 2014. Rapid detection of *Salmonella* in food samples by Polymerase Chain Reaction after a 10h pre-enrichment. *Journal of Food Safety* 34 (1):79-86.
Khan, Z., Mokaddas, E., Ahmad, S., & Burhamah, M.H.A. 2011. Isolation of *Cryptococcus magnus* and *Cryptococcus chernovii* from nasal cavities of pediatric patients with acute lymphoblastic leukemia. *Medical Mycology* 49 (4):439-443.
Kidd, S.E., Hagen, F., Tscharke, R.L., Huynh, M., Bartlett, K.H., Fyfe, M., MacDougall, L., Boekhout, T., Kwon-Chung, K.J., & Meyer, W. 2004. A rare genotype of *Cryptococcus gattii* caused the cryptococcosis outbreak on Vancouver Island (British Columbia, Canada). *Proceedings of the National Academy of Sciences of the United States of America* 101 (49):17258-17263.
Kido, N., Makimura, K., Kamegaya, C., Shindo, I., Shibata, E., Omiya, T., & Yamamoto, Y. 2012. Long-term surveillance and treatment of subclinical cryptococcosis and nasal colonization by *Cryptococcus neoformans* and *C. gattii* species complex in captive koalas (*Phascolarctes cinereus*). *Medical Mycology* 50 (3):291-298.
King, D., & Smith, L. 1985. The distribution of the European red fox (*Vulpes vulpes*) in Western Australia. *Records of the Western Australian Museum* 12 (2):197-205.
King, J.L., Duncan, C.L., & Garcelon, D.K. 2014. Status of the Santa Catalina Island fox thirteen years after its decline. *Monographs of the Western North American Naturalist* 7 (1):382-396.

King, J.M., & Bradshaw, S.D. 2010. Stress in an Island kangaroo? The Barrow Island euro, *Macropus robustus isabellinus*. *General and Comparative Endocrinology* 167 (1):60-67.
King, N.W. 2001. Herpesvirus Infections. In *Infectious Diseases of Wild Mammals*, edited by E. S. Williams & I. K. Barker. 3rd ed. Ames: Blackwell Publishing. 147-178.
Kinnear, J.E., Onus, M.L., & Bromilow, R.N. 1988. Fox control and rock-wallaby population dynamics. *Australian Wildlife Research* 15 (4):435-450.
Kirkland, P.D., Gleeson, A.B., Hawkes, R.A., Naim, H.M., & Boughton, C.R. 1989. Human infection with encephalomyocarditis virus in New South Wales. *The Medical Journal of Australia* 151 (3):176, 178.
Kitagawa, H., Sasaki, Y., & Ishihara, K. 1989. Clinical studies on canine dirofilarial hemoglobinuria: measured and calculated serum osmolalities and osmolar gap. *Nippon Juigaku Zasshi. The Japanese Journal of Veterinary Science* 51 (4):703-710.
Kitchener, D.J. 1995. Quokka, *Setonix brachyurus*. In *The Mammals of Australia*, edited by R. Strahan. Sydney: Reed Books. 401-403.
Korhola, M., Hakonen, R., Juuti, K., Edelmann, M., Kariluoto, S., Nyström, L., Sontag-Strohm, T., & Piironen, V. 2014. Production of folate in oat bran fermentation by yeasts isolated from barley and diverse foods. *Journal of Applied Microbiology*.
Kourany, M., & Vasquez, M.A. 1975. A survey to assess potential disease hazards along proposed sea level canal routes in Panama and Colombia. VIII. Survey of enterobacterial pathogens in wild caught vertebrates. *Military Medicine* 140 (1):22-25.
Koyuncu, S., Andersson, M.G., & Haggblom, P. 2010. Accuracy and sensitivity of commercial PCR-based methods for detection of *Salmonella enterica* in feed. *Applied and Environmental Microbiology* 76 (9):2815-2822.
Krasilnikov, M.A. 2000. Phosphatidylinositol-3 kinase dependent pathways: the role in control of cell growth, survival, and malignant transformation. *Biochemistry (Mosc)* 65 (1):59-67.
Kriger, K.M., & Hero, J.M. 2009. Chytridiomycosis, amphibian extinctions, and lessons for the prevention of future panzootics. *EcoHealth* 6 (1):6-10.
Krockenberger, M.B., Canfield, P.J., Barnes, J., Vogelnest, L., Connolly, J., Ley, C., & Malik, R. 2002. *Cryptococcus neoformans* var. *gattii* in the koala (*Phascolarctos cinereus*): Serological evidence for subclinical cryptococcosis. *Medical Mycology* 40 (3):273-282.
Krockenberger, M.B., Canfield, P.J., & Malik, R. 2002. Cryptococcus neoformans in the koala (*Phascolarctos cinereus*): colonization by *C n.* var. *gattii* and investigation of environmental sources. *Medical Mycology* 40 (3):263-272.
Krockenberger, M.B., Canfield, P.J., & Malik, R. 2003. *Cryptococcus neoformans* var. *gattii* in the koala (*Phascolarctos cinereus*): A review of 43 cases of cryptococcosis. *Medical Mycology* 41 (3):225-234.
Krockenberger, M.B., Stalder, K., Malik, R., & Canfield, P. 2005. Cryptococcosis in australian wildlife. *Microbiology Australia* 26 (2):69-71.
Kumar, R., Surendran, P.K., & Thampuran, N. 2008. Evaluation of culture, ELISA and PCR assays for the detection of Salmonella in seafood. *Letters in Applied Microbiology* 46 (2):221-226.
La Ragione, R., Metcalfe, H.J., Villarreal-Ramos, B., & Werling, D. 2013. Salmonella Infections in Cattle. In *Salmonella in Domestic Animals*, edited by P. A. Barrow & U. Methner. 2nd ed. Boston: CABI. 233-262.
Ladds, P. 2009. *Pathology of Australian Native Mammals*. Collingwood: CSIRO.
Ladds, P.W. 2012. Pathology of macropods. [accessed 24th August, 2014]. Available from http://www.arwh.org/sites/default/files/files-uploads/.
LaDeau, S.L., Kilpatrick, A.M., & Marra, P.P. 2007. West Nile virus emergence and large-scale declines of North American bird populations. *Nature* 447 (7145):710-713.
Lagrou, K., Van Eldere, J., Keuleers, S., Hagen, F., Merckx, R., Verhaegen, J., Peetermans, W.E., & Boekhout, T. 2005. Zoonotic transmission of *Cryptococcus neoformans* from a

magpie to an immunocompetent patient. *Journal of Internal Medicine* 257 (4):385-388.
Latney, L.V., & Wellehan, J. 2013. Selected emerging infectious diseases of squamata. *The Veterinary Clinics of North America. Exotic animal practice* 16 (2):319-338.
Le, S.Q., & Gascuel, O. 2008. An improved general amino acid replacement matrix. *Molecular Biology and Evolution* 25 (7):1307-1320.
Ledbetter, E.C., da Silva, E.C., Kim, S.G., Dubovi, E.J., & Schwark, W.S. 2012. Frequency of spontaneous canine herpesvirus-1 reactivation and ocular viral shedding in latently infected dogs and canine herpesvirus-1 reactivation and ocular viral shedding induced by topical administration of cyclosporine and systemic administration of corticosteroids. *American Journal of Veterinary Research* 73 (7):1079-1084.
Leendertz, F.H., Pauli, G., Maetz-Rensing, K., Boardman, W., Nunn, C., Ellerbrok, H., Jensen, S.A., Junglen, S., & Christophe, B. 2006. Pathogens as drivers of population declines: The importance of systematic monitoring in great apes and other threatened mammals. *Biological Conservation* 131 (2):325-337.
Leininger, J.R., Donham, K.J., & Rubino, M.J. 1978. Leprosy in a chimpanzee. Morphology of the skin lesions and characterization of the organism. *Veterinary Pathology* 15 (3):339-346.
Lester, S.J., Kowalewich, N.J., Bartlett, K.H., Krockenberger, M.B., Fairfax, T.M., & Malik, R. 2004. Clinicopathologic features of an unusual outbreak of cryptococcosis in dogs, cats, ferrets, and a bird: 38 Cases (January to July 2003). *Journal of the American Veterinary Medical Association* 225 (11):1716-1722.
Levitz, S.M. 1991. The ecology of *Cryptococcus neoformans* and the epidemiology of cryptococcosis. *Reviews of Infectious Diseases* 13 (6):1163-1169.
Lewis, J.H., Phillips, L.L., & Hann, C. 1968. Coagulation and hematological studies in primitive Australian mammals. *Comparative Biochemistry and Physiology* 25 (3):1129-1135.
Li, A.S., Pan, W.H., Wu, S.X., Hideaki, T., Guo, N.R., Shen, Y.N., Lu, G.X., Pan, R.G., Zhu, M.C., Chen, M., Shi, W.M., & Liao, W.Q. 2012. Ecological surveys of the *Cryptococcus* species complex in China. *Chinese Medical Journal* 125 (3):511-516.
Lindsay, M.D. 1995. Ecology and epidemiology of Ross River virus in Western Australia. Ph.D. diss, Department of Microbiology, University of Western Australia, Perth.
Links, I.J., Whittington, R.J., Kennedy, D.J., Grewal, A., & Sharrock, A.J. 1986. An association between encephalomyocarditis virus infection and reproductive failure in pigs. *Australian Veterinary Journal* 63 (5):150-152.
Lipsey, M.W., & Wilson, D.B. 2001. *Practical Meta-Analysis*. Vol. 49, *Applied Social Research Methods Series*. Thousand Oaks, CA: Sage Publications.
Loh, J., Collen, B., McRae, L., Carranza, T.T., Pamplin, F.A., Amin, R., & Baillie, J.E.M. 2008. Living Planet Index. In *Living Planet Report 2008*, ed C. Hails. Gland, Switzerland: WWF International. [cited 18th September, 2015] Available from http://wwf.panda.org/about_our_earth/all_publications/living_planet_report/living_planet_report_timeline/lpr_2008/.
Lorch, J.M., Muller, L.K., Russell, R.E., O'Connor, M., Lindner, D.L., & Blehert, D.S. 2013. Distribution and environmental persistence of the causative agent of white-nose syndrome, *Geomyces destructans*, in bat hibernacula of the eastern United States. *Applied and Environmental Microbiology* 79 (4):1293-1301.
Losowsky, M.S., & Leonard, P.J. 1967. Evidence of vitamin E deficiency in patients with malabsorption or alcoholism and the effects of therapy. *Gut* 8 (6):539-543.
Lozano, C.C., Sweanor, L.L., Wilson-Henjum, G., Kays, R.W., Moreno, R., VandeWoude, S., & Troyer, R.M. 2015. Identification of novel gammaherpesviruses in ocelots (*Leopardus pardalis*) and bobcats (*Lynx rufus*) in Panama and Colorado, USA. *Journal of Wildlife Diseases* 51 (4):911-915.
Lydekker, R. 1894. *A Hand-Book To The Marsupialia And Monotremata*. Edited by R. B. Sharpe. London: W.H. Allen & Co., Limited.

Mackay, R. 2008. Endotoxemia. In *Large Animal Internal Medicine*, edited by B. P. Smith. St. Louis: MOSBY. Elsevier. 711-723.
Mackenzie, J.S., Broom, A.K., Hall, R.A., Johansen, C.A., Lindsay, M.D., Phillips, D.A., Ritchie, S.A., Russell, R.C., & Smith, D.W. 1998. Arboviruses in the Australian region, 1990 to 1998. *Communicable Diseases Intelligence* 22 (6):93-99.
Mackenzie, J.S., Chua, K.B., Daniels, P.W., Eaton, B.T., Field, H.E., Hall, R.A., Halpin, K., Johansen, C.A., Kirkland, P.D., Lam, S.K., McMinn, P., Nisbet, D.J., Paru, R., Pyke, A.T., Ritchie, S.A., Siba, P., Smith, D.W., Smith, G.A., Van Den Hurk, A.F., Wang, L.F., & Williams, D.T. 2001. Emerging viral diseases of Southeast Asia and the Western Pacific. *Emerging Infectious Diseases* 7 (3):497-504.
Mackenzie, J.S., Lindsay, M.D., Coelen, R.J., Broom, A.K., Hall, R.A., & Smith, D.W. 1994. Arboviruses causing human disease in the Australasian zoogeographic region. *Archives of Virology* 136 (3-4):447-467.
Mackerras, M.J. 1959. The haematozoa of Australian mammals. *Australian Journal of Zoology* 7 (2):105-135.
MacLachlan, N.J., & Dubovi, E.J. 2011. *Fenner's Veterinary Virology*. 4th ed. Burlington: Elsevier-Academic Press.
Makanya, A.N., Haenni, B., & Burri, P.H. 2003. Morphometry and allometry of the postnatal lung development in the quokka wallaby (*Setonix brachyurus*): A light microscopic study. *Respiratory Physiology and Neurobiology* 134 (1):43-55.
Makanya, A.N., Malcolm, Sparrow, P., Charles, Warui, N., Deter, Mwangi, K., Peter, & Burri, H. 2001. Morphological analysis of the postnatally developing marsupial lung: The quokka wallaby. *Anatomical Record* 262 (3):253-265.
Makanya, A.N., Tschanz, S.A., Haenni, B., & Burri, P.H. 2007. Functional respiratory morphology in the newborn quokka wallaby (*Setonix brachyurus*). *Journal of Anatomy* 211 (1):26-36.
Malcolm, J.R., Liu, C., Neilson, R.P., Hansen, L., & Hannah, L. 2006. Global warming and extinctions of endemic species from biodiversity hotspots. *Conservation Biology* 20 (2):538-548.
Malik, R., Wigney, D.I., Muir, D.B., & Love, D.N. 1997. Asymptomatic carriage of *Cryptococcus neoformans* in the nasal cavity of dogs and cats. *Journal of Medical and Veterinary Mycology* 35 (1):27-31.
Manly, B.F.J. 2007. *Randomization, Bootstrap and Monte Carlo Methods in Biology*. 3rd ed. Boca Raton: Chapman & Hall/CRC.
Mann, P.C., Montali, R.J., & Bush, M. 1982. Mycobacterial osteomyelitis in captive marsupials. *Journal of the American Veterinary Medical Association* 181 (11):1331-1333.
Markey, B., Leonard, F., Archambault, M., Cullinane, A., & Maguire, D. 2013. *Clinical Veterinary Microbiology*. 2nd ed. Philadelphia: MOSBY Elsevier.
Martel, A., Spitzen-van der Sluijs, A., Blooi, M., Bert, W., Ducatelle, R., Fisher, M.C., Woeltjes, A., Bosman, W., Chiers, K., Bossuyt, F., & Pasmans, F. 2013. Batrachochytrium salamandrivorans sp. nov. causes lethal chytridiomycosis in amphibians. *Proceedings of the National Academy of Sciences* 110 (38):15325-15329.
Maurice, H., Nielen, M., Brocchi, E., Nowotny, N., Kassimi, L.B., Billinis, C., Loukaides, P., O'Hara, R.S., & Koenen, F. 2005. The occurrence of encephalomyocarditis virus (emcv) in european pigs from 1990 to 2001. *Epidemiology and Infection* 133 (3):547-557.
May, R.M. 1986. The cautionary tale of the black-footed ferret. *Nature* 320 (6057):13-14.
Mayberry, C., Maloney, S.K., Mitchell, J., Mawson, P.R., & Bencini, R. 2014. Reproductive implications of exposure to *Toxoplasma gondii* and *Neospora caninum* in western grey kangaroos (*Macropus fuliginosus ocydromus*). *Journal of Wildlife Diseases* 50 (2):364-368.
McAloose, D., & Newton, A.L. 2009. Wildlife cancer: A conservation perspective. *Nature Reviews Cancer* 9 (7):517-526.
McCallum, H. 2008. Tasmanian devil facial tumour disease: lessons for conservation biology. *Trends in Ecology and Evolution* 23 (11):631-637.

McCallum, H., & Dobson, A. 1995. Detecting disease and parasite threats to endangered species and ecosystems. *Trends in Ecology and Evolution* 10 (5):190-194.
McCallum, H., Tompkins, D.M., Jones, M., Lachish, S., Marvanek, S., Lazenby, B., Hocking, G., Wiersma, J., & Hawkins, C.E. 2007. Distribution and impacts of Tasmanian devil facial tumor disease. *EcoHealth* 4 (3):318-325.
McClelland, E.E., & Smith, J.M. 2011. Gender specific differences in the immune response to infection. *Archivum Immunologiae et Therapiae Experimentalis* 59 (3):203-213.
McFadden, G. 2005. Poxvirus tropism. *Nature Reviews Microbiology* 3 (3):201-213.
McGill, S., Malik, R., Saul, N., Beetson, S., Secombe, C., Robertson, I., & Irwin, P. 2009. Cryptococcosis in domestic animals in Western Australia: A retrospective study from 1995-2006. *Medical Mycology* 47 (6):625-639.
McInnes, L.M., Gillett, A., Hanger, J., Reid, S.A., & Ryan, U.M. 2011. The potential impact of native Australian trypanosome infections on the health of koalas (*Phascolarctos cinereus*). *Parasitology* 138 (7):873-883.
McInnes, L.M., Gillett, A., Ryan, U.M., Austen, J., Campbell, R.S., Hanger, J., & Reid, S.A. 2009. *Trypanosoma irwini* n. sp (Sarcomastigophora: Trypanosomatidae) from the koala (*Phascolarctos cinereus*). *Parasitology* 136 (8):875-885.
McInnes, L.M., Hanger, J., Simmons, G., Reid, S.A., & Ryan, U.M. 2011. Novel trypanosome *Trypanosoma gilletti* sp. (Euglenozoa: Trypanosomatidae) and the extension of the host range of *Trypanosoma copemani* to include the koala (*Phascolarctos cinereus*). *Parasitology* 138 (1):59-70.
McKenzie, N.L., Burbidge, A.A., Baynes, A., Brereton, R.N., Dickman, C.R., Gordon, G., Gibson, L.A., Menkhorst, P.W., Robinson, A.C., Williams, M.R., & Woinarski, J.C.Z. 2007. Analysis of factors implicated in the recent decline of Australia's mammal fauna. *Journal of Biogeography* 34 (4):597-611.
McKenzie, R.A., Fay, F.R., & Prior, H.C. 1979. Poxvirus infection of the skin of an eastern grey kangaroo. *Australian Veterinary Journal* 55 (4):188-190.
McKenzie, S., Deane, E.M., & Burnett, L. 2002. Haematology and serum biochemistry of the tammar wallaby, *Macropus eugenii*. *Comparative Clinical Pathology* 11 (4):229-237.
McKinnon, W., Weisse, C.S., Reynolds, C.P., Bowles, C.A., & Baum, A. 1989. Chronic stress, leukocyte subpopulations, and humoral response to latent viruses. *Health Psychology* 8 (4):389-402.
McLelland, D.J. 2000. Studies on the encephalomyocarditis virus in a zoological context. BSc (Veterinary), University of Sydney, Sydney.
McMurray, C.H., & Blanchflower, W.J. 1979. Application of a high-performance liquid chromatographic fluorescence method for the rapid determination of α-tocopherol in the plasma of cattle and pigs and its comparison with direct fluorescence and high-performance liquid chromatography-ultraviolet detection methods. *Journal of Chromatography A* 178 (2):525-531.
McOrist, S., Jerrett, I.V., Anderson, M., & Hayman, J. 1985. Cutaneous and respiratory tract infection with *Mycobacterium ulcerans* in two koalas (*Phascolarctos cinereus*). *Journal of Wildlife Diseases* 21 (2):171-173.
Meintker, L., Ringwald, J., Rauh, M., & Krause, S.W. 2013. Comparison of automated differential blood cell counts from Abbott Sapphire, Siemens Advia 120, Beckman Coulter DxH 800, and Sysmex XE-2100 in normal and pathologic samples. *American Journal of Clinical Pathology* 139 (5):641-650.
Merchant, I.A., & Packer, R.A. 1967. *Veterinary Bacteriology and Virology*. 7th ed. Ames, IA: Iowa State University Press.
Metre, D.C.V., & Mackay, R.J. 2015. Localization and Differentiation of Neurologic Diseases. In *Large Animal Internal Medicine*, edited by B. P. Smith. 5th ed. St. Louis: Elsevier, Mosby. 107-130.
Meyer, W., Castañeda, A., Jackson, S., Huynh, M., Castañeda, E., Arechavala, A., Davel, G., Rodero, L., Perrotta, D., Lazera, M., Pereira-Igreja, R., Wanke, B., Mendes-Giannini, M.J., Melhem, M.S.C., Henning-Vainstein, M., Diaz, M.C., Restrepo, A., Huérfano, S.,

Samayoa, B., Logeman, H., Martirez, R.L., Olivares, L.R.C., Contreras-Peres, C., Tovar, J.F.V., Bustamante, B., Torres-Rodriquez, J., Morera, Y., & Calvo, B. 2003. Molecular typing of IberoAmerican *Cryptococcus neoformans* isolates. *Emerging Infectious Diseases* 9 (2):189-195.

Meyer, W., Gilgado, F., Ngamskulrungroj, P., Trilles, L., Hagen, F., Castañeda, E., & Boekhout, T. 2011. Molecular Typing of the *Cryptococcus neoformans/Cryptococcus gattii* Species Complex. In *Cryptococcus: From Human Pathogen to Model Yeast*, edited by J. Heitman, T. R. Kozel, K. J. Kwon-Chung, J. R. Perfect & A. Casadevall. Washington, DC: ASM Press. 327-357.

Michael, S.A., & Sangster, C.R. 2010. Pulmonary mycobacteriosis caused by *Mycobacterium intracellulare* in a Tasmanian devil (*Sarcophilus harrisii*). *Australian Veterinary Journal* 88 (7):280-282.

Migaki, G., Gunnels, R., & Casey, H. 1978. Pulmonary cryptococcosis in an Atlantic bottlenosed dolphin (*Tursiops truncatus*). *Laboratory Animal Science* 28 (5):603-606.

Miller, D.L., Radi, Z.A., Stiver, S.L., & Thornhill, T.D. 2004. Cutaneous and pulmonary mycosis in green anacondas (*Euncectes murinus*). *Journal of Zoo and Wildlife Medicine* 35 (4):557-561.

Miller, D.S., Mitchel, G.F., Biggs, B., McCracken, H., Myroniuk, P., & Hewish, M. 2000. Agglutinating antibodies to *Toxoplasma gondii* in sera from captive eastern barred bandicoots in Australia. *Journal of Wildlife Diseases* 36 (2):213-218.

Miller, M.A., Ehlers, K., Dubey, J.P., & Van Steenbergh, K. 1992. Outbreak of toxoplasmosis in wallabies on an exotic animal farm. *Journal of Veterinary Diagnostic Investigation* 4 (4):480-483.

Mishra, R., Sahoo, P.K., Mishra, S., Achary, K.G., Dwibedi, B., Kar, S.K., & Satapathy, A.K. 2014. Bancroftian filariasis: Circulating B-1 cells decreased in microfilaria carriers and correlate with immunoglobulin M levels. *Parasite Immunology* 36 (5):207-217.

Mitchell, T.G., & Perfect, J.R. 1995. Cryptococcosis in the era of AIDS--100 years after the discovery of *Cryptococcus neoformans*. *Clinical Microbiology Reviews* 8 (4):515-548.

Mohler, V.L., Izzo, M.M., & House, J.K. 2009. Salmonella in Calves. *Veterinary Clinics of North America - Food Animal Practice* 25 (1):37-54.

Moloney, B. 2001. Encephalomyocarditis virus and mortalities in captive wombats. Paper read at *Proceedings of the Australian Society for Veterinary Pathology Annual Conference*, at Melbourne.

Molter, C.M., Zuba, J.R., & Papendick, R. 2014. *Cryptococcus gattii* osteomyelitis and compounded itraconazole treatment failure in a pesquet's parrot (*Psittrichas fulgidus*). *Journal of Zoo and Wildlife Medicine* 45 (1):127-133.

Monga, D.P., & Garg, D.N. 1980. Ovine pulmonary infection caused by *Rhodotorula rubra*. *Mykosen* 23 (4):208-211.

Moore, J. 1903. Tuberculosis in an Australian possum. *Veterinary Journal* 57:283.

Moore, K.W., O'Garra, A., De Waal Malefyt, R., Vieira, P., & Mosmann, T.R. 1993. Interleukin-10. *Annual Review of Immunology* 11:165-190.

Mora, M., Napolitano, C., Ortega, R., Poulin, E., & Pizarro-Lucero, J. 2015. Feline immunodeficiency virus and feline leukemia virus infection in free-ranging guignas (*Leopardus guigna*) and sympatric domestic cats in human perturbed landscapes on Chiloe Island, Chile. *Journal of Wildlife Diseases* 51 (1):199-208.

Moreno, S., Beltrán, J.F., Cotilla, I., Kuffner, B., Laffite, R., Jordán, G., Ayala, J., Quintero, C., Jiménez, A., Castro, F., Cabezas, S., & Villafuerte, R. 2007. Long-term decline of the European wild rabbit (*Oryctolagus cuniculus*) in south-western Spain. *Wildlife Research* 34 (8):652-658.

Moseby, K. 2012. National Recovery Plan for the Plains Mouse (*Pseudomys australis*). South Australia. Department of Environment, Water and Natural Resources. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.

Moss, B. 2001. *Poxviridae*: the viruses and their replication. In *Fields Virology*, edited by D. M. Knipe & P. M. Howley. 4th ed. Philadelphia, PA: Lippincott, Williams and Wilkins. 2849-2883.

Mugisha, L., Leendertz, F.H., Opuda-Asibo, J., Olobo, J.O., & Ehlers, B. 2010. A novel herpesvirus in the sanctuary chimpanzees on Ngamba Island in Uganda. *Journal of Medical Primatology* 39 (1):71-76.

Müller, G., Gröters, S., Siebert, U., Rosenberger, T., Driver, J., König, M., Becher, P., Hetzel, U., & Baumgärtner, W. 2003. Parapoxvirus infection in harbor seals (*Phoca vitulina*) from the German North Sea. *Veterinary Pathology* 40 (4):445-454.

Muller, M.J. 1995. Veterinary arbovirus vectors in Australia - A retrospective. *Veterinary Microbiology* 46 (1-3):101-116.

Munday, B.L. 1976. Diseases of native land mammals of Australia. In *Australian Fauna Management and Disease. Refresher Course for Veterinarians Proceedings No. 29*, *Sydney*, 11–18 Postgraduate Committee in Veterinary Science. University of Sydney.

Munday, B.L. 1978. Marsupial diseases. In *Fauna - Part B. Proceedings No. 36 of Course for Veterinarians (The J.D. Stewart Course for 1978)*, *Mosman*, *6-10 February 1978*, 335-386: Postgraduate Committee in Veterinary Science. University of Sydney.

Munday, B.L. 1988. Marsupial diseases. In *Proceedings No. 104. Australian Wildlife. Refresher Course for Veterinarians*, *Sydney*, 299-365: Postgraduate Committee in Veterinary Science. University of Sydney.

Munson, L., Terio, K.A., Kock, R., Mlengeya, T., Roelke, M.E., Dubovi, E., Summers, B., Sinclair, A.R.E., & Packer, C. 2008. Climate extremes promote fatal co-infections during canine distemper epidemics in African lions. *PLoS One* 3 (6).

Myint, M.S., Johnson, Y.J., Tablante, N.L., & Heckert, R.A. 2006. The effect of pre-enrichment protocol on the sensitivity and specificity of PCR for detection of naturally contaminated Salmonella in raw poultry compared to conventional culture. *Food Microbiology* 23 (6):599-604.

Nagornaya, S.S., Babich, T.V., Podgorsky, V.S., Beharav, A., Nevo, E., & Wasser, S.P. 2003. Yeast interslope divergence in soils and plants of "Evolution Canyon", Lower Nahal Oren, Mount Carmel, Israel. *Israel Journal of Plant Sciences* 51 (1):55-57.

Naish, S., Hu, W., Nicholls, N., Mackenzie, J.S., McMichael, A.J., Dale, P., & Tong, S. 2006. Weather variability, tides and Barmah Forest virus disease in the Gladstone region, Australia. *Environmental Health Perspectives* 114 (5):678-683.

Nataro, J.P., Bopp, C.A., Fields, P.I., Kaper, J.B., & Strockbine, N.A. 2011. Escherichia, Shigella, and Salmonella. In *Manual of Clinical Microbiology*, edited by J. Versalovic, K. C. Carroll, G. Funke, J. H. Jorgensen, M. L. Landry & D. W. Warnock. 10th Edition ed. Washington, D.C.: ASM Press. 603-626.

Nelson, R.W., & Couto, C.G. 2009. *Small Animal Internal Medicine*. 4th ed. St. Louis: MOSBY Elsevier.

Nevitt, B.N., Langan, J.N., Adkesson, M.J., Delaney, M.A., Rubin, D.A., Muhlbauer, M.C., & Colegrove, K.M. 2013. Multifocal *Cryptococcus neoformans* var. *neoformans*: Infection, treatment, and monitoring by serial computed tomography in a schmidt's red-tailed guenon (*Cercopithecus ascanius schmidti*). *Journal of Zoo and Wildlife Medicine* 44 (3):728-736.

Ni, J., Wen, X., Yao, J., Chang, H.C., Yin, Y., Zhang, M., Xie, S., Chen, M., Simons, B., Chang, P., di Sant'Agnese, A., Messing, E.M., & Yeh, S. 2005. Tocopherol-associated protein suppresses prostate cancer cell growth by inhibition of the phosphoinositide 3-kinase pathway. *Cancer Res* 65 (21):9807-9816.

Nielsen, K., Cox, G.M., Wang, P., Toffaletti, D.L., Perfect, J.R., & Heitman, J. 2003. Sexual cycle of *Cryptococcus neoformans* var. *grubii* and virulence of congenic a and alpha isolates. *Infection and Immunity* 71 (9):4831-4841.

Nielsen, L.R. 2013. Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of *Salmonella* Dublin in cattle. *Veterinary Microbiology* 162 (1):1-9.

Nielsen, N.O., Simonsen, P.E., Magnussen, P., Magesa, S., & Friis, H. 2006. Cross-sectional relationship between HIV, lymphatic filariasis and other parasitic infections in adults in coastal northeastern Tanzania. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 100 (6):543-550.

Nishikawa, M.M., Lazera, M.S., Barbosa, G.G., Trilles, L., Balassiano, B.R., Macedo, R.C.L., Bezerra, C.C.F., Pérez, M.A., Cardarelli, P., & Wanke, B. 2003. Serotyping of 467 *Cryptococcus neoformans* isolates from clinical and environmental sources in Brazil: analysis of host and regional patterns. *Journal of Clinical Microbiology* 41 (1):73-77.

Niven, B. 1970. Mathematics of populations of the quokka, *Setonix brachyurus* (Marsupialia) I. A simple deterministic model for quokka populations. *Australian Journal of Zoology* 18 (2):209-214.

Nosanchuk, J.D., Shoham, S., Fries, B.C., Shapiro, D.S., Levitz, S.M., & Casadevall, A. 2000. Evidence of zoonotic transmission of *Cryptococcus neoformans* from a pet cockatoo to an immunocompromised patient. *Annals of Internal Medicine* 132 (3):205-208.

Nowakiewicz, A., Zieba, P., Ziolkowska, G., Gnat, S., Muszynska, M., Tomczuk, K., Majer Dziedzic, B., Ulbrych, L., & Troscianczyk, A. 2016. Free-living species of carnivorous mammals in poland: red fox, beech marten, and raccoon as a potential reservoir of *Salmonella*, *Yersinia*, *Listeria* spp. and coagulase-positive *Staphylococcus*. *PLoS One* 11 (5):e0155533.

Noyes, H.A., Stevens, J.R., Teixeira, M., Phelan, J., & Holz, P. 1999. A nested PCR for the ssrRNA gene detects *Trypanosoma binneyi* in the platypus and *Trypanosoma* sp. in wombats and kangaroos in Australia. *International Journal for Parasitology* 29 (2):331-339.

O'Brien, A.D., Scher, I., Campbell, G.H., MacDermott, R.P., & Formal, S.B. 1979. Susceptibility of CBA/N mice to infection with *Salmonella* typhimurium: Influence of the X-linked gene controlling B lymphocyte function. *Journal of Immunology* 123 (2):720-724.

O'Regan, N.L., Steinfelder, S., Venugopal, G., Rao, G.B., Lucius, R., Srikantam, A., & Hartmann, S. 2014. *Brugia malayi* microfilariae induce a regulatory monocyte/macrophage phenotype that suppresses innate and adaptive immune responses. *PLoS Neglected Tropical Diseases* 8 (10).

O'Reilly, L.M., & Daborn, C.J. 1995. The epidemiology of *Mycobacterium bovis* infections in animals and man: A review. *Tubercle and Lung Disease* 76 (SUPPL. 1):1-46.

Obendorf, D.L., & Munday, B.L. 1990. Toxoplasmosis in wild eastern barred bandicoots, *Perameles gunnii*. In *Bandicoots and Bilbies*, edited by J. H. Seebeck, P. R. Brown, R. L. Wallis & C. M. Kemper. Sydney, NSW: Surrey Beatty and Sons Pty Ltd. 193-197.

Oberste, M.S., Gotuzzo, E., Blair, P., Nix, W.A., Ksiazek, T.G., Comer, J.A., Rollin, P., Goldsmith, C.S., Olson, J., & Kochel, T.J. 2009. Human febrile illness caused by encephalomyocarditis virus infection, Peru. *Emerging Infectious Diseases* 15 (4):640-646.

Ochsenbein, A.F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H., & Zinkernagel, R.M. 1999. Control of early viral and bacterial distribution and disease by natural antibodies. *Science* 286 (5447):2156-2159.

Oliveira, C.J.B., Carvalho, L.F.O.S., & Garcia, T.B. 2006. Experimental airborne transmission of *Salmonella* agona and *Salmonella* typhimurium in weaned pigs. *Epidemiology and Infection* 134 (1):199-209.

Olsen, I., Barletta, R.G., & Thoen, C.O. 2010. Mycobacterium. In *Pathogenesis of Baterial Infections in Animals*, edited by C. L. Gyles, J. F. Prescott, G. Songer & C. O. Thoen. 4th ed. Ames: Wiley-Blackwell.

Pacioni, C., Robertson, I.D., Maxwell, M., van Weenen, J., & Wayne, A.F. 2013. Hematologic characteristics of the Woylie (*Bettongia penicillata ogilbyi*). *Journal of Wildlife Diseases* 49 (4):816-830.

Packer, W.C. 1969. Observations on the behavior of the marsupial *Setonix brachyurus* (Quoy and Gaimard) in an enclosure. *Journal of Mammalogy* 50 (1):8-20.

Paddle, R. 2012. The thylacine's last straw: epidemic disease in a recent mammalian extinction. *Australian Zoologist* 36 (1):75-92.
Padgett, D.A., & Glaser, R. 2003. How stress influences the immune response. *Trends in Immunology* 24 (8):444-448.
Palmer, C., Taylor, R., & Burbidge, A. 2003. Recovery plan for the Golden Bandicoot *Isoodon auratus* and Golden-backed Tree-rat *Mesembriomys macrurus* 2004 - 2009. Darwin. Northern Territory Department of Infrastructure Planning and Environment. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.
Pan, S., Thompson, R.C.A., Grigg, M.E., Sundar, N., Smith, A., & Lymbery, A.J. 2012. Western Australian marsupials are multiply infected with genetically diverse strains of *Toxoplasma gondii*. *PLoS One* 7 (9).
Papadimitriou, J.M., & Ashman, R.B. 1972. A poxvirus in a marsupial papilloma. *Journal of General Virology* 16 (1):87-89.
Paparini, A., Irwin, P.J., Warren, K., McInnes, L.M., de Tores, P., & Ryan, U.M. 2011. Identification of novel trypanosome genotypes in native Australian marsupials. *Veterinary Parasitology* 183 (1-2):21-30.
Pappas, P.G. 2013. Cryptococcal infections in non-HIV-infected patients. *Transactions of the American Clinical and Climatological Association* 124:61-79.
Parameswaran, N., O'Handley, R.M., Grigg, M.E., Fenwick, S.G., & Thompson, R.C.A. 2009. Seroprevalence of *Toxoplasma gondii* in wild kangaroos using an ELISA. *Parasitology International* 58 (2):161-165.
Park, B.J., Wannemuehler, K.A., Marston, B.J., Govender, N., Pappas, P.G., & Chiller, T.M. 2009. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* 23 (4):525-530.
Parsons, S.K., Bull, C.M., & Gordon, D.M. 2010. Low prevalence of *Salmonella enterica* in Australian wildlife. *Environmental Microbiology Reports* 2 (5):657-659.
Patton, S., Johnson, S.L., Loeffler, D.G., Wright, B.G., & Jensen, J.M. 1986. Epizootic of toxoplasmosis in kangaroos, wallabies, and potaroos: possible transmission via domestic cats. *Journal of the American Veterinary Medical Association* 189 (9):1166-1169.
Pavey, C. 2006. National Recovery Plan for the Greater Bilby (*Macrotis lagotis*). Darwin. Northern Territory Department of Natural Resources, Environment and the Arts. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.
Payne, K.L., Vitali, S.D., Eden, P.A., Vaughan, R.J., & Krockenberger, M.B. 2005. Incidence of cryptococcal disease at Perth zoo-an epidemiological investigation. Paper read at *Health in a Shrinking World: Ecology, Management and Conservation. Proceedings of the Wildlife Disease Assocation International Conference*, at Qld, Australia.
Pearse, A.M., & Swift, K. 2006. Allograft theory: Transmission of devil facial-tumour disease. *Nature* 439 (7076):549.
Pearson, D.J. 2013. Recovery plan for five species of rock wallabies: Black- footed rock wallaby (*Petrogale lateralis*), Rothschild rock wallaby (*Petrogale rothschildi*), Short-eared rock wallaby (*Petrogale brachyotis*), Monjon (*Petrogale burbidgei*) and Nabarlek (*Petrogale concinna*) 2012-2022. Wildlife Management Program No. 55. Perth. Department of Parks and Wildlife. [accessed 20th September, 2015]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.
Pedersen, A.B., Jones, K.E., Nunn, C.L., & Altizer, S. 2007. Infectious diseases and extinction risk in wild mammals. *Conservation Biology* 21 (5):1269-1279.
Peet, R.L., Dickson, J., & Nickels, D. 1982. *Mycobacterium intracellulare* infection in wallabies. *Australian Veterinary Journal* 58 (5):215-215.

Pejcic-Karapetrovic, B., Gurnani, K., Russell, M.S., Finlay, B.B., Sad, S., & Krishnan, L. 2007. Pregnancy impairs the innate immune resistance to *Salmonella* typhimurium leading to rapid fatal infection. *Journal of Immunology* 179 (9):6088-6096.

Pellet, P.E., & Roizman, B. 2007. The Family Herpesviridae: A brief introduction. In *Fields Virology*, edited by D. M. Knipe, P. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman & S. E. Straus. 5th ed. New York, N.Y.: Lippincott-Williams and Wilkins. 2479-2499.

Perry, D.H. 1973. Some notes on the decline and subsequent recovery of mammal populations in the south-west. *The Western Australian Naturalist* 12:128-130.

Peter, W., & Feiler, A. 1994. Eine neue Bovidenart aus Vietnam und Cambodia (Mammalia: Ruminantia). *Zool. Abh. Staatliches Mus. Tierkd. Dresden* 48:169-176.

Peterson, A.T., Ortega-Huerta, M.A., Bartley, J., Sanchez-Cordero, V., Soberon, J., Buddemeier, R.H., & Stockwell, D.R.B. 2002. Future projections for Mexican faunas under global climate change scenarios. *Nature* 416 (6881):626-629.

Petti, C.A. 2007. Detection and identification of microorganisms by gene amplification and sequencing. *Clinical Infectious Diseases* 44 (8):1108-1114.

Pfeiffer, T., & Ellis, D. 1997. Additional *Eucalyptus* hosts for *Cryptococcus neoformans* var. gattii. Paper read at *13th Congress of the International Society for Human and Animal Mycology (ISHAM). Australia*.

Pfeiffer, T.J., & Ellis, D.H. 1993. Serotypes of Australian environmental and clinical isolates of *Cryptococcus neoformans*. *Journal of Medical and Veterinary Mycology* 31 (5):401-404.

Philips, A., & Driessen, M. 2008. Strategy for managing wildlife diseases in the Tasmanian wilderness World Heritage Area. Hobart, TAS: Wildlife and Marine Conservation Section, Biodiversity Conservation Branch. Department of Primary Industries and Water. [accessed 15th July, 2013]. Available from http://www.dpiw.tas.gov.au/inter.nsf/WebPages/LJEM-79T3DP?open.

Phillott, A.D., Grogan, L.F., Cashins, S.D., McDonald, K.R., Berger, L., & Skerratt, L.F. 2013. Chytridiomycosis and seasonal mortality of tropical stream-associated frogs 15 years after introduction of *Batrachochytrium dendrobatidis*. *Conservation Biology* 27 (5):1058-1068.

Pickering, J., & Norris, C.A. 1996. New evidence concerning the extinction of the endemic murid *Rattus macleari* Thomas, 1887 from Christmas Island, Indian Ocean. *Australian Mammalogy* 19:19-26.

Pinto, J., Bonacic, C., Hamilton-West, C., Romero, J., & Lubroth, J. 2008. Climate change and animal diseases in South America. *OIE Revue Scientifique et Technique* 27 (2):599-613.

Plimmer, H. 1914. Report of the deaths which occurred in the Zoological Gardens during 1913, together with a list of the blood parasites found during the year. Proceedings of the Zoological Society of London:181-190. [accessed August 23rd 2011]. Available from http://www.biodiversitylibrary.org/.

Plyusnin, A., Beaty, B.J., Elliott, R.M., Goldbach, R., Kormelink, R., Lundkvist, A., Schmaljohn, C.S., & Tesh, R.B. 2012. Bunyaviridae. In *Virus Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*, edited by A. M. Q. King, M. J. Adams, E. B. Carstens & E. J. Lefkowitz. San Diego: Elsevier Academic Press. 725–741.

Polednik, L., Rehulka, J., Kranz, A., Polednikova, K., Hlavac, V., & Kazihnitkova, H. 2008. Physiological responses of over-wintering common carp (*Cyprinus carpio*) to disturbance by Eurasian otter (*Lutra lutra*). *Fish Physiology and Biochemistry* 34 (3):223-234.

Poole, H.L., Mukaromah, L., Kobryn, H.T., & Fleming, P.A. 2014. Spatial analysis of limiting resources on an island: diet and shelter use reveal sites of conservation importance for the Rottnest Island quokka. *Wildlife Research* 41 (6):510-521.

Pope, J.H. 1959. A virus of the encephalomyocarditis group from a water-rat, *Hydromys chrysogaster*, in North Queensland. *Australian Journal of Experimental Biology and Medical Science* 37:117-124.

Portas, T., Fletcher, D., Spratt, D., Reiss, A., Holz, P., Stalder, K., Devlin, J., Taylor, D., Dobroszczyk, D., & Manning, A.D. 2014. Health evaluation of free-ranging eastern bettongs (*Bettongia gaimardi*) during translocation for reintroduction in Australia. *Journal of Wildlife Diseases* 50 (2):210-223.

Portas, T.J. 2010. Toxoplasmosis in macropodids: a review. *Journal of Zoo and Wildlife Medicine* 41 (1):1-6.

Poth, T., Seibold, M., Werckenthin, C., & Hermanns, W. 2010. First report of a *Cryptococcus magnus* infection in a cat. *Medical Mycology* 48 (7):1000-1004.

Potkay, S. 1977. Diseases of australiasian marsupials. In *The Biology of Marsupials*, edited by D. Hunsaker. New York: Academic Press Inc. 456-506.

Potter, A.S., Reid, S.A., & Fenwick, S.G. 2011. Prevalence of *Salmonella* in fecal samples of western grey kangaroos (*Macropus fuliginosus*). *Journal of Wildlife Diseases* 47 (4):880-887.

Poulet, H., Minke, J., Pardo, M.C., Juillard, V., Nordgren, B., & Audonnet, J.C. 2007. Development and registration of recombinant veterinary vaccines: The example of the canarypox vector platform. *Vaccine* 25 (30 SPEC. ISS.):5606-5612.

Prociv, P. 1987. Parasites of australian flying-foxes (Chiroptera: pteropodidae). *Australian Mammalogy* (10):107-110.

Purvis, A., Gittleman, J.L., Cowlishaw, G., & Mace, G.M. 2000. Predicting extinction risk in declining species. *Proceedings of the Royal Society B: Biological Sciences* 267 (1456):1947-1952.

Pye, R.J., Woods, G.M., & Kreiss, A. 2015. Devil Facial Tumor Disease. *Veterinary Pathology*.

Quesada, R.J., Heard, D.J., Aitken-Palmer, C., Hall, N., Conley, K.J., Childress, A.L., & Wellehan, J.F.X. 2011. Detection and phylogenetic characterization of a novel herpesvirus from the trachea of two stranded common loons (*Gavia immer*). *Journal of Wildlife Diseases* 47 (1):233-239.

Quinn, P.J., Carter, M.E., Markey, B.K., & Carter, G.R. 1994. *Clinical Veterinary Microbiology*. Spain: WOLFE.

Quinn, P.J., Markey, B.K., Leonard, F.C., FitzPatrick, E.S., & Fanning, S. 2016. *Concise Review of Veterinary Microbiology*. 2nd ed. Oxford: Wiley-Blackwell.

Quinn, P.J., Markey, B.K., Leonard, F.C., FitzPatrick, E.S., Fanning, S., & Hartigan, P.J. 2011. *Veterinary Microbiology and Microbial Disease*. 2nd ed. Oxford: Wiley-Blackwell.

Radostits, O.M., Gay, C.C., Hinchcliff, K.W., & Constable, P.D. 2010. *Veterinary Medicine: A textbook of the diseases of cattle, sheep, goats, pigs and horses*. 10th ed. Philadelphia: Saunders, ELSEVIER.

Rappaport, F., Konforti, N., & Navon, B. 1956. A new enrichment medium for certain salmonellae. *Journal of Clinical Pathology* 9 (3):261-266.

Rastogi, N., Legrand, E., & Sola, C. 2001. The mycobacteria: an introduction to nomenclature and pathogenesis. *Revue scientifique et technique De L Office International Des Epizooties* 20 (1):21.

Reddacliff, G.L., Hartley, W.J., Dubey, J.P., & Cooper, D.W. 1993. Pathology of experimentally-induced, acute toxoplasmosis in macropods. *Australian Veterinary Journal* 70 (1):4-6.

Reddacliff, L.A. 2012. Viral Chorioretinitis of Kangaroos. In *Fowler's Zoo and Wild Animal Medicine Current Therapy 7*, edited by R. E. Miller & M. Fowler. St. Louis, MO: Elsevier-Saunders. 390-394.

Reddacliff, L.A., Kirkland, P.D., Hartley, W.J., & Reece, R.L. 1997. Encephalomyocarditis virus infections in an Australian zoo. *Journal of Zoo and Wildlife Medicine* 28 (2):153-157.

Reece, R.L., & Hartley, W.J. 1994. The pathology registry and some interesting cases. In *Proceedings 233. Wildlife. The TG Hungerford Refresher Course for Veterinarians.*, *Sydney*, 217-233: Post Graduate Foundation in Veterinary Science.

Reese, T.A. 2016. Coinfections: Another variable in the herpesvirus latency-reactivation dynamic. *Journal of Virology* 90 (12):5534-5537.

Reese, T.A., Wakeman, B.S., Choi, H.S., Hufford, M.M., Huang, S.C., Zhang, X., Buck, M.D., Jezewski, A., Kambal, A., Liu, C.Y., Goel, G., Murray, P.J., Xavier, R.J., Kaplan, M.H.,

Renne, R., Speck, S.H., Artyomov, M.N., Pearce, E.J., & Virgin, H.W. 2014. Helminth infection reactivates latent gamma-herpesvirus via cytokine competition at a viral promoter. *Science* 345 (6196):573-577.

Reid, S.A., Husein, A., Partoutomo, S., & Copeman, D.B. 2001. The susceptibility of two species of wallaby to infection with *Trypanosoma evansi*. *Australian Veterinary Journal* 79 (4):285-288.

Renter, D.G., Gnad, D.P., Sargeant, J.M., & Hygnstrom, S.E. 2006. Prevalence and serovars of *Salmonella* in the feces of free-ranging white-tailed deer (*Odocoileus virginianus*) in Nebraska. *Journal of Wildlife Diseases* 42 (3):699-703.

Rheinbaben, F., Gebel, J., Exner, M., & Schmidt, A. 2007. Environmental resistance, disinfection, and sterilization of poxviruses. In *Poxviruses*, edited by A. Mercer, A. Schmidt & O. Weber: Birkhäuser Basel. 397-405.

Rhyan, J.C., & Spraker, T.R. 2010. Emergence of diseases from wildlife reservoirs. *Veterinary Pathology* 47 (1):34-39.

Richter, V., & Freegard, C. 2009a. Standard Operating Procedure No. 12.1: Permanent marking of vertebrates using microchips. Perth, WA: Department of Parks and Wildlife. Government of Western Australia. [accessed 13th June, 2012]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/96-monitoring/standards/99-standard-operating-procedures.

Richter, V., & Freegard, C. 2009b. Standard Operating Procedure No. 12.3: Semi-permanent marking of mammals using ear tags. Perth, WA: Department of Parks and Wildlife. Government of Western Australia. [accessed 13th June, 2012]. Available from http://www.dpaw.wa.gov.au/plants-and-animals/96-monitoring/standards/99-standard-operating-procedures.

Ride, W.D.L. 1957. Protemnodon parma (waterhouse) and the classification of related wallabies (*Protemnodon*, *Thyloqale*, and *Setonix*). *Proceedings of the Zoological Society of London* 128 (3):327-346.

Rippey, E., Hislop, M.C., & Dodd, J. 2003. Reassessment of the vascular flora of Rottnest Island. *Journal of the Royal Society of Western Australia* (86):7-23.

Rippey, M.E., & Hobbs, R.J. 2003. The effects of fire and quokkas (*Setonix brachyurus*) on the vegetation of Rottnest Island, Western Australia. *Journal of the Royal Society of Western Australia* 86 (2):49-60.

Risbey, D.A., Calver, M.C., Short, J., Bradley, J.S., & Wright, I.W. 2000. The impact of cats and foxes on the small vertebrate fauna of Heirisson Prong, Western Australia. II. A field experiment. *Wildlife Research* 27 (3):223-235.

Roache, M. 2011. The action plan for threatened Australian macropods 2011-2021. Sydney. WWF-Australia. [accessed January 21, 2013]. Available from http://www.wwf.org.au/news_resources/resource_library/?2940/The-action-plan-for-threatened-Australian-macropods-2011-2021.

Robert, K.A., & Schwanz, L.E. 2013. Monitoring the health status of free-ranging tammar wallabies using hematology, serum biochemistry, and parasite loads. *The Journal of Wildlife Management*.

Robinson, A.J., & Kerr, P.J. 2001. Poxvirus Infections. In *Infectious Diseases of Wild Mammals*, edited by E. S. Williams & I. K. Barker. 3rd ed. Ames: Blackwell Publishing. 179-201.

Rock, D., Lokensgard, J., Lewis, T., & Kutish, G. 1992. Characterization of dexamethasone-induced reactivation of latent bovine herpesvirus 1. *Journal of Virology* 66 (4):2484-2490.

Roizman, B., & Pellet, P. 2001. The family *Herpesviridae*: A brief introduction. In *Fields Virology*, edited by D. M. Knipe & P. M. Howley. 4th ed. Philadelphia, PA: Lippincott Williams, and Wilkins. 2381–2398.

Rose, K., Kirkland, P.D., Claasens, R., Blumstein, D., & Cooper, D.W. 2000. An epizootic of sudden death in tammar wallabies (*Macropus eugenii*). In *Proceedings of Wildlife Disease Association (Australasian Section) Annual Conference.*, *University of Canberra Field Station*, 166-170.

Rostal, M.K., Evans, A.L., Solberg, E.J., & Arnemo, J.M. 2012. Hematology and serum chemistry reference ranges of free-ranging moose (*Alces alces*) in Norway. *Journal of Wildlife Diseases* 48 (3):548-559.
Rothwell, T.L., Keep, J.M., Xu, F.N., & Middleton, D.J. 1984. Poxvirus in marsupial skin lesions. *Australian Veterinary Journal* 61 (12):409-410.
Rucker, R.B., Morris, J., & Fascetti, A.J. 2008. Vitamins. In *Clinical Biochemistry of Domestic Animals*, edited by J. J. Kaneko, J. W. Harvey & M. L. Bruss. San Diego: Academic Press, Elsevier. 695-730.
Ruiz-Piña, H.A., Puc-Franco, M.A., Flores-Abuxapqui, J., Vado-Solís, I., & Cárdenas-Marrufo, M.F. 2002. Isolation of *Salmonella enterica* and serologic reactivity to *Leptospira interrogans* in opossums (*Didelphis virginiana*) from Yucatán, México. *Revista do Instituto de Medicina Tropical de Sao Paulo* 44 (4):235-237.
Runyon, E.H. 1959. Anonymous mycobacteria in pulmonary disease. *The Medical Clinics of North America* 43 (1):273-290.
Russell, R.C. 2002. Ross river virus: Ecology and distribution. In *Annual Review of Entomology*.
Russell, R.C., & Dwyer, D.E. 2000. Arboviruses associated with human disease in Australia. *Microbes and Infection* 2 (14):1693-1704.
Sacher, R.A., Luban, N.L.C., Ameti, D.I., Friend, S., Schreiber, G.B., Murphy, E.L., & for the Retrovirus Epidemiology Donor Study, G. 1999. Low prevalence of flower cells in U.S.A. blood donors infected with human T-lymphotrophic virus types I and II. *British Journal of Haematology* 105 (3):758-763.
Sadleir, R.M.F.S. 1959. Comparative aspects of the ecology and physiology of Rottnest and Byford populations of the quokka (*Setonix brachyurus*, Quoy and Gaimard). Honours diss., University of Western Australia, Perth.
Salgame, P., Yap, G.S., & Gause, W.C. 2013. Effect of helminth-induced immunity on infections with microbial pathogens. *Nature Immunology* 14 (11):1118-1126.
Salkowski, C.A., Bartizal, K.F., Balish, M.J., & Balish, E. 1987. Colonization and pathogenesis of *Cryptococcus neoformans* in gnotobiotic mice. *Infection and Immunity* 55 (9):2000-2005.
Samuel, J.L. 1989. Viral diseases of Australian mammals and birds. In *Conference on Virology for Australian Veterinarians*, *Brisbane*, 295-312: Veterinary Science Postgraduate Committee, University of Queensland.
Sanchez, S., Hofacre, C.L., Lee, M.D., Maurer, J.J., & Doyle, M.P. 2002. Animal sources of salmonellosis in humans. *Journal of the American Veterinary Medical Association* 221 (4):492-497.
Santos, R.L., Tsolis, R.M., B√ § umler, A.J., & Adams, L.G. 2002. Hematologic and serum biochemical changes in *Salmonella* ser Typhimurium-infected calves. *American Journal of Veterinary Research* 63 (8):1145-1150.
Saunders, D.A., & De Rebeira, P. 2009. A case study of the conservation value of a small tourist resort Island: Birds of Rottnest Island, Western Australia 1905-2007. *Pacific Conservation Biology* 15 (1):11-31.
Schloegel, L.M., Hero, J.M., Berger, L., Speare, R., McDonald, K., & Daszak, P. 2006. The decline of the sharp-snouted say frog (*Taudactylus acutirostris*): The first documented case of extinction by infection in a free-ranging wildlife species? *EcoHealth* 3 (1):35-40.
Schmidt, K.L., Chin, E.H., Shah, A.H., & Soma, K.K. 2009. Cortisol and corticosterone in immune organs and brain of European starlings: developmental changes, effects of restraint stress, comparison with zebra finches. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 297 (1):42-51.
Schneider, C.A., Rasband, W.S., & Eliceiri, K.W. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* 9 (7):671-675.
Schoon, H.A., Schoon, D., Kirpal, G., Richter, E., Gerdes, J., Weiss, R., & Dressen, W. 1993. Enzootic *Mycobacterium avium* infection in captive Parma wallabies (*Macropus*

parma) with unusual spinal cord manifestations. *Journal of Comparative Pathology* 108 (3):311-316.
Scott, M.E. 1988. The impact of infection and disease on animal populations: implications for conservation biology. *Conservation Biology* 2 (1):40-56.
Seaman, J.T., Boulton, J.G., & Carrigan, M.J. 1986. Encephalomyocarditis virus disease of pigs associated with a plague of rodents. *Australian Veterinary Journal* 63 (9):292-294.
Seebeck, J.H., & Rose, R.W. 1989. Potoroidae. In *Fauna Of Australia* edited by D. W. Walton & B. J. Richardson. Canberra: Australian Government Publishing Service.
Semler, B.L., & Ertel, K.J. 2010. Picornaviruses: Molecular Biology. In *Desk Encyclopedia of General Virology*, edited by B. W. J. Mahy & M. H. V. V. Regenmortel. Oxford, UK: Elsevier-Academic Press. 565-576.
Semnani, R.T., Keiser, P.B., Coulibaly, Y.I., Keita, F., Diallo, A.A., Traore, D., Diallo, D.A., Doumbo, O.K., Traore, S.F., Kubofcik, J., Klion, A.D., & Nutman, T.B. 2006. Filaria-induced monocyte dysfunction and its reversal following treatment. *Infection and Immunity* 74 (8):4409-4417.
Semnani, R.T., Venugopal, P.G., Leifer, C.A., Mostböck, S., Sabzevari, H., & Nutman, T.B. 2008. Inhibition of TLR3 and TLR4 function and expression in human dendritic cells by helminth parasites. *Blood* 112 (4):1290-1298.
Sharman, G. 1954. The relationships of the quokka (*Setonix brachyurus*). *The Western Australian Naturalist* 4:159-168.
Sharman, G.B. 1955a. Studies on marsupial reproduction. 2. The oestrous cycle of *Setonix brachyurus*. . *Australian Journal of Zoology* 3 (1):44-55.
Sharman, G.B. 1955b. Studies on marsupial reproduction. 3. Normal and delayed pregnancy in *Setonix brachyurus*. *Australian Journal of Zoology* 3 (1):56-70.
Sharman, G.B. 1961. The mitotic chromosomes of Marsupials and their bearing on taxonomy and phylogeny. *Australian Journal of Zoology* 9 (1):38-60.
Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., & Boonstra, R. 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia* 166 (4):869-887.
Shield, J. 1964. A breeding season difference in two populations of the Australian macropod marsupial (*Setonix brachyurus*). *Journal of Mammalogy* 45 (4):616-625.
Shield, J. 1968. Reproduction of the quokka, *Setonix brachyurus*, in captivity. *Journal of Zoology* 155:427-444.
Shield, J. 1971. A seasonal change in blood cell volume of the Rottnest Island quokka, *Setonix brachyurus*. *Journal of Zoology* 165:343-354.
Shield, J.W. 1958. Aspects of field ecology of the Quokka (*Setonix brachyurus* Quoy and Gaimard) Ph.D. diss., Faculty of Science, University of Western Australia, Perth.
Shimoyama, M. 1991. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the lymphoma study group (1984-87). *British Journal of Haematology* 79 (3):428-437.
Shimoyama, M., Minato, K., Tobinai, K., Nagai, M., Setoya, T., Takenaka, T., Ishihara, K., Watanabe, S., Hoshino, H., Miwa, M., Kinoshita, M., Okabe, S., Fukushima, N., & Inada, N. 1983. Atypical adult T-cell leukemia-lymphoma: diverse clinical manifestations of adult T-cell leukemia-lymphoma. *Japanese Journal of Clinical Oncology* 13 Suppl 2:165-187.
Short, J., & Smith, A. 1994. Mammal decline and recovery in Australia. *Journal of Mammalogy* 75 (2):288-297.
Shortridge, G.C. 1909. An account of the geographical distribution of the marsupials and monotremes of South-West Australia, having special reference to the specimens collected during the Balston expedition of 1904-1907. *Proceedings of the General Meetings for Scientific Business of the Zoological Society of London* (May-December):803-848.
Shultz, M.T., & Kitaysky, A.S. 2008. Spatial and temporal dynamics of corticosterone and corticosterone binding globulin are driven by environmental heterogeneity. *General and Comparative Endocrinology* 155 (3):717-728.

Siddle, H.V., Kreiss, A., Eldridge, M.D.B., Noonan, E., Clarke, C.J., Pyecroft, S., Woods, G.M., & Belov, K. 2007. Transmission of a fatal clonal tumor by biting occurs due to depleted MHC diversity in a threatened carnivorous marsupial. *Proceedings of the National Academy of Sciences of the United States of America* 104 (41):16221-16226.

Simon, E., Paul, J.L., Atger, V., Simon, A., & Moatti, N. 1998. Erythrocyte antioxidant status in asymptomatic hypercholesterolemic men. *Atherosclerosis* 138 (2):375-381.

Sinclair, E.A. 1998. Morphological variation among populations of the quokka, *Setonix brachyurus* (Macropodidae : Maruspialia), in Western Australia. *Australian Journal of Zoology* 46 (5):439-449.

Sinclair, E.A. 2001. Phylogeographic variation in the quokka, *Setonix brachyurus* (Marsupialia: Macropodidae): Implications for conservation. *Animal Conservation* 4 (4):325-333.

Sipiczki, M. 2013. Detection of yeast species also occurring in substrates associated with animals and identification of a novel dimorphic species in *Verbascum* flowers from Georgia. *Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology* 103 (3):567-576.

Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K.R., Phillott, A.D., Hines, H.B., & Kenyon, N. 2007. Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. *EcoHealth* 4 (2):125-134.

Skinner, M.A. 2010. Fowlpox Virus and Other Avipoxviruses. In *Desk Encyclopedia of Animals and Bacterial Virology*, edited by B. W. J. Mahy & M. H. V. v. Regenmortel. San Diego, CA: Academic Press-Elsevier. 397-406.

Smith, A., Clark, P., Averis, S., Lymbery, A.J., Wayne, A.F., Morris, K.D., & Thompson, R.C.A. 2008. Trypanosomes in a declining species of threatened Australian marsupial, the brush-tailed bettong *Bettongia penicillata* (Marsupialis:Potoroidae). *Parasitology* 135:1329-1335.

Smith, B.P. 2008. *Large Animal Internal Medicine*. 4th ed. St. Louis: MOSBY Elsevier.

Smith, J.A., Wellehan Jr, J.F.X., Pogranichniy, R.M., Childress, A.L., Landolfi, J.A., & Terio, K.A. 2008. Identification and isolation of a novel herpesvirus in a captive mob of eastern grey kangaroos (*Macropus giganteus*). *Veterinary Microbiology* 129 (3-4):236-245.

Smith, J.H., Folse, D.S., Long, E.G., Christie, J.D., Crouse, D.T., Tewes, M.E., Gatson, A.M., Ehrhardt, R.L., File, S.K., & Kelly, M.T. 1983. Leprosy in wild armadillos (*Dasypus novemcinctus*) of the Texas gulf coast: Epidemiology and mycobacteriology. *RES Journal of the Reticuloendothelial Society* 34 (2):75-88.

Smith, K.F., Sax, D.F., & Lafferty, K.D. 2006. Evidence for the role of infectious disease in species extinction and endangerment. *Conservation Biology* 20 (5):1349-1357.

Sokal, R.R., & Michener, C.D. 1958. A statistical method for evaluating systematic relationships. *University of Kansas Science Bulletin* (38):1409-1438.

Songer, J.G., & Post, K.W. 2005. *Veterinary Microbiology: Bacterial and Fungal Agents of Animal Disease*. 1st ed. St. Louis: Elsevier Saunders.

Sorrell, T.C., Chen, S.C., Ruma, P., Meyer, W., Pfeiffer, T.J., Ellis, D.H., & Brownlee, A.G. 1996. Concordance of clinical and environmental isolates of *Cryptococcus neoformans* var. *gattii* by random amplification of polymorphic DNA analysis and PCR fingerprinting. *Journal of Clinical Microbiology* 34 (5):1253-1260.

Spalding, M.G., & Forrester, D.J. 1993. Disease Monitoring of Free-Ranging and Released Wildlife. *Journal of Zoo and Wildlife Medicine* 24 (3):271-280.

Speare, R. 1988a. Clinical assessment, diseases and management of the orphaned joey. In *Australian Wildlife: The John Keep Refresher Course for Veterinarians*, *Sydney*, 211-298: Postgraduate Committee in Veterinary Science, University of Sydney.

Speare, R. 1988b. Clinical assessment, diseases and management of the orphaned macropod PY. In *Proceedings 104. Australian Wildlife. Refresher Course for Veterinarians*, *Sydney*, 211-296: Postgraduate Committee in Veterinary Science. University of Sydney.

Speck, S.H., & Ganem, D. 2010. Viral latency and its regulation: lessons from the gamma-herpesviruses. *Cell Host & Microbe* 8 (1):100-115.
Spencer, A.J., & Canfield, P.J. 1996. Lymphoid neoplasia in the koala (*Phascolarctos cinereus*): A review and classification of 31 cases. *Journal of Zoo and Wildlife Medicine* 27 (3):303-314.
Sperber, I. 1960. Excretion. In *Biology and Comparative Physiology of BIRDS*, edited by A. J. Marshall. New York, NY: Academic Press inc. 469-492.
Spier, S.J., Smith, B.P., Cullor, J.S., Olander, H.J., Roden, L.D., & Dilling, G.W. 1991. Persistent experimental Salmonella dublin intramammary infection in dairy cows. *Journal of Veterinary Internal Medicine* 5 (6):341-350.
Spratt, D.M., & Presidente, P.J. 1978. Disease problems in captive tammar wallabies (*Macropus eugenii*). In *Wildlife Disease Association (Australasian Section) Annual Meeting*, *Sydney*: Wildlife Disease Assocation.
Staff, M., Musto, J., Hogg, G., Janssen, M., & Rose, K. 2012. Salmonellosis outbreak traced to playground sand, Australia, 2007-2009. *Emerging Infectious Diseases* 18 (7):1159-1162.
Staib, F., Seibold, M., Antweiler, E., Fröhlich, B., Weber, S., & Blisse, A. 1987. The brown colour effect (BCE) of *Cryptococcus neoformans* in the diagnosis, control and epidemiology of *C. neoformans* infections in AIDS patients. *Zentralblatt fur Bakteriologie Mikrobiologie und Hygiene - Abt. 1 Orig. A* 266 (1-2):167-177.
Stalder, K., Vaz, P.K., Gilkerson, J.R., Baker, R., Whiteley, P., Ficorilli, N., Tatarczuch, L., Portas, T., Skogvold, K., Anderson, G.A., & Devlin, J.M. 2015. Prevalence and clinical significance of herpesvirus infection in populations of Australian marsupials. *PLoS One* 10 (7):e0133807.
Stalder, K.J. 2013. Herpesviruses in Australian marsupials. M.Sc. diss, Faculty of Veterinary Science, The University of Melbourne, Melbourne.
Stanley, N.F. 1975. The Ord River Dam of tropical Australia. In *Man-made Lakes and Human Health*, edited by N. F. Stanley & M. P. Alpers. London: Academic Press. 103-112.
Stanley, N.F., & Choo, S.B. 1964. Studies of Arboviruses in Western Australia- serological epidemiology. *Bulletin of the World Health Organization* 30:221-226.
Stanley, N.F., & Leak, P.J. 1963. The serologic epidemiology of reovirus infection with special reference to the Rottnest Island quokka (*Setonix brachyurus*). *American Journal of Epidemiology* 78 (1):82-88.
Stanley, N.F., Leak, P.J., Grieve, G.M., & Perret, D.H. 1964. The ecology and epidemiology of reovirus. *Australian Journal of Experimental Biology and Medical Science* 42:373-384.
Stanley, N.F., & Mackenzie, J.S. 1983. Microbiology studies on Rottnest Island. *Journal of the Royal Society of Western Australia* 66:31-34.
Stanton, J.J., Nofs, S.A., Zachariah, A., Kalaivannan, N., & Ling, P.D. 2014. Detection of elephant endotheliotropic herpesvirus infection among healthy Asian elephants (*Elephas maximus*) in South India. *Journal of Wildlife Diseases* 50 (2):279-287.
Steenbergen, J.N., & Casadevall, A. 2000. Prevalence of *Cryptococcus neoformans* var. *neoformans* (serotype D) and *Cryptococcus neoformans* var. *grubii* (serotype A) isolates in New York City. *Journal of Clinical Microbiology* 38 (5):1974-1976.
Stevenson, C.A. 2011. Ecological impacts of Australian ravens on bush bird communities on Rottnest island. Hons. diss., School of Biological Sciences and Biotechnology, Murdoch University, Perth.
Stirrat, S.C. 2003. Body condition and blood chemistry of agile wallabies (*Macropus agilis*) in the wet-dry tropics. *Wildlife Research* 30 (1):59-67.
Stokes, V.L., & Norman, M.A. 2010. Threatened fauna species management plans for ALCOA'S bauxite mining operations in the Jarrah forest. *Unpublished Report*.
Storr, G.M. 1964. Studies on marsupial nutrition IV. Diet of the quokka, *Setonix brachyurus* (Quoy & Gaimard), on Rottnest Island, Western Australia. *Australian Journal of Biological Sciences* 17:469-481.

Storr, G.M. 1965. Notes on Bald Island and the adjacent mainland. *The Western Australian Naturalist* 9 (8):187-196.
Su, L., Su, C.W., Qi, Y., Yang, G., Zhang, M., Cherayil, B.J., Zhang, X., & Shi, H.N. 2014. Coinfection with an intestinal helminth impairs host innate immunity against *Salmonella enterica* serovar Typhimurium and exacerbates intestinal inflammation in mice. *Infection and Immunity* 82 (9):3855-3866.
Suedmeyer, W.K., & Johnson, G. 2007. Survey of neoplasia in red kangaroos (*Macropus rufus*), 1992-2002, in a zoological collection. *Journal of Zoo and Wildlife Medicine* 38 (2):231-239.
Superina, M., & Mera y Sierra, R.L. 2008. Hematology and serum chemistry values in captive and wild pichis, *Zaedyus pichiy* (Mammalia, Dasypodidae). *Journal of Wildlife Diseases* 44 (4):902-910.
Sykes, J.E. 2014. Pediatric feline upper respiratory disease. *Veterinary Clinics of North America: Small Animal Practice* 44 (2):331-342.
Sykes, J.E., & Malik, R. 2012. Cryptococcosis. In *Infectious Diseases of the Dog and Cat*, edited by C. E. Green. 4th ed. St. Louis, MO: Saunders ELSEVIER. 621-634.
Tachibana, N., Okayama, A., Ishihara, S., Shioiri, S., Murai, K., Tsuda, K., Goya, N., Matsuo, Y., Essex, M., Stuver, S., & Mueller, N. 1992. High HTLV-I proviral DNA level associated with abnormal lymphocytes in peripheral blood flow asymptomatic carriers. *International Journal of Cancer* 51 (4):593-595.
Tam, M.A., Rydström, A., Sundquist, M., & Wick, M.J. 2008. Early cellular responses to Salmonella infection: Dendritic cells, monocytes, and more. *Immunological Reviews* 225 (1):140-162.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28 (10):2731-2739.
Tanaka, S., & Mannen, K. 2003. Effect of mild stress in mice latently infected pseudorabies virus. *Experimental Animals* 52 (5):383-386.
Tarlinton, R., Meers, J., Hanger, J., & Young, P. 2005. Real-time reverse transcriptase PCR for the endogenous koala retrovirus reveals an association between plasma viral load and neoplastic disease in koalas. *Journal of General Virology* 86 (3):783-787.
Tate, G.H.H. 1948. Results of the Archbold Expeditions N0. 59. Studies on the anatomy and phylogeny of the Macropodidae (Marsupialia). *Bulleting of the American Museum of Natural History* 91.
Teare, J.A. 2002. *Reference ranges for physiological values in captive wildlife*. International Species Information System (ISIS) [accessed October 13th 2014]. Available from http://library.murdoch.edu.au/database/isis/.
Telfer, S., Lambin, X., Birtles, R., Beldomenico, P., Burthe, S., Paterson, S., & Begon, M. 2010. Species interactions in a parasite community drive infection risk in a wildlife population. *Science* 330 (6001):243-246.
Tell, L.A., Nichols, D.K., Fleming, W.P., & Bush, M. 1997. Cryptococcosis in tree shrews (*Tupaia tana* and *Tupaia minor*) and elephant shrews (*Macroscelides proboscides*). *Journal of Zoo and Wildlife Medicine* 28 (2):175-181.
Tennant, B.C., & Center, S.A. 2008. Hepatic Function. In *Clinical Biochemistry of Domestic Animals (Sixth Edition)*, edited by J. J. Kaneko, J. W. Harvey & M. L. Bruss. San Diego: Academic Press. 379-412.
Tesh, R.B., & Wallace, G.D. 1978. Observations on the natural history of encephalomyocarditis virus. *American Journal of Tropical Medicine and Hygiene* 27 (1 I):133-143.
Tezuka, K., Xun, R., Tei, M., Ueno, T., Tanaka, M., Takenouchi, N., & Fujisawa, J.I. 2014. An animal model of adult T-cell leukemia: Humanized mice with HTLV-1-specific immunity. *Blood* 123 (3):346-355.
Thogmartin, W.E., King, R.A., McKann, P.C., Szymanski, J.A., & Pruitt, L. 2012. Population-level impact of white-nose syndrome on the endangered Indiana bat. *Journal of Mammalogy* 93 (4):1086-1098.

Thomas, C.D., Cameron, A., Green, R.E., Bakkenes, M., Beaumont, L.J., Collingham, Y.C., Erasmus, B.F.N., Ferreira De Siqueira, M., Grainger, A., Hannah, L., Hughes, L., Huntley, B., Van Jaarsveld, A.S., Midgley, G.F., Miles, L., Ortega-Huerta, M.A., Peterson, A.T., Phillips, O.L., & Williams, S.E. 2004. Extinction risk from climate change. *Nature* 427 (6970):145-148.

Thompson, C.K., Godfrey, S.S., & Thompson, R.C.A. 2014. Trypanosomes of Australian mammals: A review. *International Journal for Parasitology: Parasites and Wildlife* 3 (2):57-66.

Thompson, G.R., Bengis, R.G., & Brown, C.C. 2001. Picornavirus Infections. In *Infectious Diseases of Wild Mammals*, edited by E. S. Williams & I. K. Barker. 3rd ed. Ames: Blackwell Publishing. 119-130.

Thompson, R.C.A., Lymbery, A.J., & Smith, A. 2010. Parasites, emerging disease and wildlife conservation. *International Journal for Parasitology* 40 (10):1163-1170.

Thomson, C., Hann, C., & Johnson, C. 2012. *Vetinary Neuroanatomy: A Clinical Approach*. Edinburgh: SAUNDERS, Elsevier.

Thorne, E.T., & Williams, E.S. 1988. Disease and endangered species: the black-footed ferret as a recent example. *Conservation Biology* 2 (1):66-74.

Thrall, M.A. 2012a. Classification of and Diagnostic Approach to Anemia. In *Veterinary Hematology And Clinical Chemistry*, edited by T. Mary Anna, W. Glade, W. A. Robin & W. C. Terry. 2nd Ed. ed. Ames, Iowa: Wiley-Blackwell. 75-80.

Thrall, M.A. 2012b. Classification of and Diagnostic Approach to Polycythemia. In *Veterinary Hematology And Clinical Chemistry`*, edited by T. Mary Anna, W. Glade, W. A. Robin & W. C. Terry. 2nd Ed. ed. Ames, Iowa: Wiley-Blackwell. 114-117.

Thrall, M.A. 2012c. Erythrocyte Morphology. In *Veterinary Hematology And Clinical Chemistry*, edited by T. Mary Anna, W. Glade, W. A. Robin & W. C. Terry. 2nd Ed. ed. Ames, Iowa: Wiley-Blackwell. 61-74.

Thrall, M.A. 2012d. Regenerative Anemia. In *Veterinary Hematology And Clinical Chemistry*, edited by T. Mary Anna, W. Glade, W. A. Robin & W. C. Terry. 2nd Ed. ed. Ames, Iowa: Wiley-Blackwell. 87-113.

Throughton, E. 1941. *Furred Animals of Australia*. Sydney: Angus and Robertson.

Timm, S.F., Munson, L., Summers, B.A., Terio, K.A., Dubovi, E.J., Rupprecht, C.E., Kapil, S., & Garcelon, D.K. 2009. A suspected canine distemper epidemic as the cause of a catastrophic decline in Santa Catalina Island foxes (*Urocyon littoralis catalinae*). *Journal of Wildlife Diseases* 45 (2):333-343.

Tomlinson, A.J., Chambers, M.A., Wilson, G.J., McDonald, R.A., & Delahay, R.J. 2013. Sex-related heterogeneity in the life-history correlates of mycobacterium bovis infection in european badgers (*Meles meles*). *Transboundary and Emerging Diseases* 60 (SUPPL1):37-45.

Troyer, R.M., Beatty, J.A., Stutzman-Rodriguez, K.R., Carver, S., Lozano, C.C., Lee, J.S., Lappin, M.R., Riley, S.P., Serieys, L.E., Logan, K.A., Sweanor, L.L., Boyce, W.M., Vickers, T.W., McBride, R., Crooks, K.R., Lewis, J.S., Cunningham, M.W., Rovnak, J., Quackenbush, S.L., & VandeWoude, S. 2014. Novel gammaherpesviruses in North American domestic cats, bobcats, and pumas: identification, prevalence, and risk factors. *Journal of Virology* 88 (8):3914-3924.

Truman, R.W., Shannon, E.J., Hagstad, H.V., Hugh-Jones, M.E., Wolff, A., & Hastings, R.C. 1986. Evaluation of the origin of *Mycobacterium leprae* infections in the wild armadillo, *Dasypus novemcinctus*. *American Journal of Tropical Medicine and Hygiene* 35 (3):588-593.

Tseng, M., Fleetwood, M., Reed, A., Gill, V.A., Harris, R.K., Moeller, R.B., Lipscomb, T.P., Mazet, J.A.K., & Goldstein, T. 2012. Mustelid herpesvirus-2, a novel herpes infection in northern sea otters (*Enhydra lutris kenyoni*). *Journal of Wildlife Diseases* 48 (1):181-185.

VanDevanter, D.R., Warrener, P., Bennett, L., Schultz, E.R., Coulter, S., Garber, R.L., & Rose, T.M. 1996. Detection and analysis of diverse herpesviral species by consensus primer PCR. *Journal of Clinical Microbiology* 34 (7):1666-1671.

Vassiliadis, P., Trichopoulos, D., Kalandidi, A., & Xirouchaki, E. 1978. Isolation of salmonellae from sewage with a new procedure of enrichment. *Journal of Applied Bacteriology* 44 (2):233-239.

Vaughan, R. 2008. Health and Disease Status of Australia's most critically endangered mammal the Gilbert's potoroo (*Potorous gilbertii*). Ph.D. diss., School of Veterinary & Biological Sciences, Murdoch University, Perth.

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., & Malik, R. 2007. Cryptococcosis in Gilbert's and long-nosed potoroo. *Journal of Zoo and Wildlife Medicine* 38 (4):567-573.

Vaughan, R.J., Warren, K.S., Mills, J.S., Palmer, C., Fenwick, S., Monaghan, C.L., & Friend, A.J. 2009. Hematological and serum biochemical reference values and cohort analysis in the gilbert's potoroo (*Potorous Gilbertii*). *Journal of Zoo and Wildlife Medicine* 40 (2):276-288.

Vaz, P., Whiteley, P.L., Wilks, C.R., Browning, G.F., Gilkerson, J.R., Ficorilli, N., & Devlin, J.M. 2012. Detection of a second novel gammaherpesvirus in a free-ranging koala (*Phascolarctos cinereus*). *Journal of Wildlife Diseases* 48 (1):226-229.

Vaz, P., Whiteley, P.L., Wilks, C.R., Duignan, P.J., Ficorilli, N., Gilkerson, J.R., Browning, G.F., & Devlin, J.M. 2011. Detection of a novel gammaherpesvirus in koalas (*Phascolarctos cinereus*). *Journal of Wildlife Diseases* 47 (3):787-791.

Vaz, P.K., Motha, J., McCowan, C., Ficorilli, N., Whiteley, P.L., Wilks, C.R., Hartley, C.A., Gilkerson, J.R., Browning, G.F., & Devlin, J.M. 2013. Isolation and characterization of a novel herpesvirus from a free-ranging eastern grey kangaroo (*Macropus giganteus*). *Journal of Wildlife Diseases* 49 (1):143-151.

Vogelnest, L. 2015. Marsupialia (Marsupials). In *Fowler's Zoo and Wild Animal Medicine*, edited by R. E. Miller & E. F. Murray. St. Louis, Missouri: Elsevier/Saunders. 255-274.

Vogelnest, L., & Portas, T.J. 2010. Macropods. In *Medicine of Australian Mammals*, edited by L. Vogelnest & R. Woods. Collingwood, Victoria: CSIRO Publishing. 133-225.

Vogelnest, L., Stewart, S., & Sangster, C. 2012. Poxvirus infection outbreak in common ringtails (*Pseudocheirus peregrinus*). *Australian Veterinary Journal* 90 (4):143-145.

Vogelnest, L., & Woods, R. 2010. *Medicine of Australian Mammals*. Collingwood: CSRIO Publishing.

Vrbova, L., Stephen, C., Kasman, N., Boehnke, R., Doyle-Waters, M., Chablitt-Clark, A., Gibson, B., FitzGerald, M., & Patrick, D.M. 2010. Systematic review of surveillance systems for emerging zoonoses. *Transboundary and Emerging Diseases* 57 (3):154-161.

Vredenburg, V.T., Knapp, R.A., Tunstall, T.S., & Briggs, C.J. 2010. Dynamics of an emerging disease drive large-scale amphibian population extinctions. *Proceedings of the National Academy of Sciences* 107 (21):9689-9694.

Wahid, S. 1962. On a new species of breinlia (Nematoda: Filarioidea) from *Macropus brachyurus*. *Journal of Helminthology* 36:207-210

Wallace, H.A., Jacobson, A., & Hammack, T.S. 2009. *Salmonella: in Bacteriological Analytical Manual (BAM)*. U.S. Food and Drug Administration [accessed February 13th 2013]. Available from http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm.

Walter, C.T., & Barr, J.N. 2011. Recent advances in the molecular and cellular biology of bunyaviruses. *Journal of General Virology* 92 (Pt 11):2467-2484.

Ware, W.A. 2009. Heartworm Disease. In *Small Animal Internal Medicine*, edited by R. W. Nelson & C. G. Couto. 4th ed. St. Louis: Mosby ELSEVIER. 169-183.

Watanabe, S., Maeda, K., Suzuki, K., Ueda, N., Iha, K., Taniguchi, S., Shimoda, H., Kato, K., Yoshikawa, Y., Morikawa, S., Kurane, I., Akashi, H., & Mizutani, T. 2010. Novel betaherpesvirus in bats. *Emerging Infectious Diseases* 16 (6):986-988.

Watson, P.J., & Bunch, S.E. 2009. Hepatobiliary and Exocrine Pancreatic Disorders. In *Small Animal Internal Medicine*, edited by R. W. Nelson & C. G. Couto. 4th ed. St. Louis: Mosby ELSEVIER. 485-606.

Watson, P.R., Paulin, S.M., Jones, P.W., & Wallis, T.S. 2000. Interaction of *Salmonella* serotypes with porcine macrophages in vitro does not correlate with virulence. *Microbiology* 146 (7):1639-1649.

Wattiau, P., Boland, C., & Bertrand, S. 2011. Methodologies for *Salmonella enterica* subsp. *enterica* subtyping: gold standards and alternatives. *Applied and Environmental Microbiology* 77 (22):7877-7885.

Weaver, S.C., & Reisen, W.K. 2010. Present and future arboviral threats. *Antiviral Research* 85 (2):328-345.

Webber, C.E., & Whalley, J.M. 1978. Widespread occurrence in Australian marsupials of neutralizing antibodies to a herpesvirus from a parma wallaby. *Australian Journal of Experimental Biology and Medical Science* 56 (3):351-357.

Weber, D.K., Danielson, K., Wright, S., & Foley, J.E. 2002. Hematology and serum biochemistry values of dusky-footed wood rat (*Neotoma fuscipes*). *Journal of Wildlife Diseases* 38 (3):576-582.

Wedderburn, L., Tighe, H., Greenwood, B.M., Marsh, K., Whittle, H.C., Brown, J., & Seidelin, P. 1984. T-cell control of Epstein-Barr virus-infected B cells is lost during *P. falciparum* malaria. *Nature* 312 (5993):449-450.

Weese, J.S. 2011. Bacterial Enteritis in Dogs and Cats: Diagnosis, Therapy, and Zoonotic Potential. *Veterinary Clinics of North America - Small Animal Practice* 41 (2):287-309.

Weigl, R. 2005. *Longevity of Mammals in Captivity; from the Living Collections of the World*. Sttutgart: Kleine Senckenberg-Reihe 48.

Weiser, G. 2012. Interpretation of leukocyte responses in disease. In *Veterinary Hematology And Clinical Chemistry*, edited by T. Mary Anna, W. Glade, W. A. Robin & W. C. Terry. 2nd Ed. ed. Ames, Iowa: Wiley-Blackwell. 127-139.

Weiss, G., & Goodnough, L.T. 2005. Anemia of chronic disease. *The New England Journal of Medicine* 352 (10):1011-1023.

Weiss, S.H., Blaser, M.J., Paleologo, F.P., Black, R.E., McWhorter, A.C., Asbury, M.A., Carter, G.P., Feldman, R.A., & Brenner, D.J. 1986. Occurrence and distribution of serotypes of the Arizona subgroup of *Salmonella* strains in the United States from 1967 to 1976. *Journal of Clinical Microbiology* 23 (6):1056-1064.

Weiss, S.W. 1996. Lipomatous tumors. *Monographs in Pathology* 38:207-239.

Wellehan, J.F., Jr., Childress, A.L., Marschang, R.E., Johnson, A.J., Lamirande, E.W., Roberts, J.F., Vickers, M.L., Gaskin, J.M., & Jacobson, E.R. 2009. Consensus nested PCR amplification and sequencing of diverse reptilian, avian, and mammalian orthoreoviruses. *Veterinary Microbiology* 133 (1-2):34-42.

Welles, E.G., Hall, A.S., & Carpenter, D.M. 2009. Canine complete blood counts: A comparison of four in-office instruments with the ADVIA 120 and manual differential counts. *Veterinary Clinical Pathology* 38 (1):20-29.

Wells, S.K., Gutter, A.E., Soike, K.F., & Baskin, G.B. 1989. Encephalomyocarditis virus-epizootic in a zoological collection. *Journal of Zoo and Wildlife Medicine* 20 (3):291-296.

Western Australian Herbarium. 1998. *FloraBase—the Western Australian Flora v. 2.9*. Department of Parks and Wildlife [accessed 20th February, 2014. Available from http://florabase.dpaw.wa.gov.au/.

White, S.R. 1952. The occurrence of the quokka in the south-west. *The Western Australian Naturalist* 3:101-103.

White, T.J., Bruns, T., Lee, S., & Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols. A Guide to Methods and Applications*, edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press, Inc. 315-322.

Whittington, R.J. 1993. Diseases of Monotremes. In *Zoo and Wild Animal Medicine. Current Therapy 3*, edited by M. E. Fowler. Philadelphia: WB Saunders. 269-275.

Widén, F., das Neves, C.G., Ruiz-Fons, F., W. Reid, H., Kuiken, T., Gavier-Widen, D., & F. Kaleta, E. 2012. Herpesvirus Infections. In *Infectious Diseases of Wild Mammals and Birds in Europe*, edited by D. Gavier-Widén, J. P. Duff & A. Meredith. Oxford, UK: Wiley-Blackwell Publishing. 3-36.

Wilcox, R.S., Vaz, P., Ficorilli, N.P., Whiteley, P.L., Wilks, C.R., & Devlin, J.M. 2011. Gammaherpesvirus infection in a free-ranging eastern grey kangaroo (*Macropus giganteus*). *Australian Veterinary Journal* 89 (1-2):55-57.

Wilkins, W., Waldner, C., Rajic, A., McFall, M., Muckle, A., & Mainar-Jaime, R.C. 2010. Comparison of bacterial culture and real-time PCR for the detection of Salmonella in grow-finish pigs in Western Canada using a Bayesian approach. *Zoonoses and Public Health* 57 Suppl 1:115-120.

Wilks, C.R., Kefford, B., & Callinan, R.B. 1981. Herpesvirus as a cause of fatal disease in Australian wallabies. *Journal of Comparative Pathology* 91 (3):461-465.

Williams, A.A.J., Karoly, D.J., & Tapper, N. 2001. The sensitivity of Australian fire danger to climate change. *Climatic Change* 49 (1-2):171-191.

Williams, S.E., Bolitho, E.E., & Fox, S. 2003. Climate change in Australian tropical rainforests: An impending environmental catastrophe. *Proceedings of the Royal Society B: Biological Sciences* 270 (1527):1887-1892.

Winkler, M.T., Doster, A., Sur, J.H., & Jones, C. 2002. Analysis of bovine trigeminal ganglia following infection with bovine herpesvirus 1. *Veterinary Microbiology* 86 (1-2):139-155.

Wirth, F., & Goldani, L.Z. 2012a. Epidemiology of rhodotorula: An emerging pathogen. *Interdisciplinary Perspectives on Infectious Diseases* 2012.

Wirth, F., & Goldani, L.Z. 2012b. Experimental Rhodotorulosis infection in rats. *APMIS* 120 (3):231-235.

Wobeser, G.A. 2007. *Disease in Wild Animals Investigation and Management*. 2nd ed. Berlin, Germany: Springer.

Woinarski, J.C.Z., Burbidge, A.A., & Harrison, P. 2014. *The action plan for Australian mammals 2012*. Melbourne: CSIRO Publishing.

Woolf, B. 1955. On estimating the relation between blood group and disease. *Ann Hum Genet* 19 (4):251-253.

Woolford, L., Bennett, M.D., Sims, C., Thomas, N., Friend, J.A., Nicholls, P.K., Warren, K.S., & O'Hara, A.J. 2009. Prevalence, emergence, and factors associated with a viral papillomatosis and carcinomatosis syndrome in wild, reintroduced, and captive western barred bandicoots (*Perameles bougainville*). *EcoHealth*:1-12.

Wyatt, K.B., Campos, P.F., Gilbert, M.T.P., Kolokotronis, S.-O., Hynes, W.H., DeSalle, R., Daszak, P., Ross, D.E.M., & Greenwood, A.D. 2008. Historical mammal extinction on Christmas Island (Indian Ocean) correlates with introduced infectious disease. *PLoS One* 3 (11).

Wynne, C.D.L., & Leguet, B. 2004. Detour behavior in the Quokka (*Setonix brachyurus*). *Behavioural Processes* 67 (2):281-286.

Yadav, M., Stanley, N.F., & Waring, H. 1972. The microbial flora of the gut of the pouch-young and the pouch of a marsupial, *Setonix brachyurus*. *Microbiology* 70 (3):437-442.

Yamaguchi, K., Kiyokawa, T., Nakada, K., Yul, L.S., Asou, N., Ishii, T., Sanada, I., Seiki, M., Yoshida, M., & Matutes, E. 1988. Polyclonal integration of HTLV-I proviral DNA in lymphocytes from HTLV-I seropositive individuals: an intermediate state between the healthy carrier state and smouldering ATL. *British Journal of Haematology* 68 (2):169.

Yang, R., Fenwick, S., Potter, A., Elliot, A., Power, M., Beveridge, I., & Ryan, U. 2012. Molecular characterization of Eimeria species in macropods. *Experimental Parasitology* 132 (2):216-221.

Yeatman, G.J., & Groom, C.J. 2012. National Recovery Plan for the woylie (*Bettongia penicillata*). Wildlife Management Program No. 51. Perth. Department of Parks and Wildlife. [accessed 12th August, 2013]. Available from

http://www.dpaw.wa.gov.au/plants-and-animals/threatened-species-and-communities/197-approved-recovery-plans.
Yen, P.K.F. 1983. Filariasis in the Australian marsupial, *Setonix brachyurus* (Quokka). Ph.D. diss., Department of Microbiology, University of Western Australia, Perth.
Young, L., & Deane, E. 2006. A longitudinal study of changes in blood leukocyte numbers in the tammar wallaby, *Macropus eugenii*. *Comparative Clinical Pathology* 15 (2):63-69.
Zheltikova, T.M., Glushakova, A.M., & Alesho, N.A. 2011. The yeast community associated with the digestive tract of the German cockroach *Blattella germanica*. *Meditsinskaia Parazitologiia I Parazitarnye Bolezni* (2):18-22.
Ziegler, C., Käufer-Weiss, I., & Zahner, H. 1991. On the pathogenesis of anaemia and leukopenia in filarial (*Litomosoides carinii*) infection of *Mastomys natalensis*. *Zentralblatt fur Veterinarmedizin. Reihe B. Journal of veterinary medicine. Series B* 38 (2):123-134.
Zimbro, M.J., Power, D.A., Miller, S.M., Wilson, G.E., & Johnson, J.A. 2009. Difco™ & BBL™ Manual. Manual of Microbiological Culture Media. 2nd ed. Sparks: Becton, Dickinson and Company.
Zimmerman, J. 1994. Encephalomyocarditis. In *Handbook of Zoonoses, Section B: Viral*, edited by G. W. Beran & H. S. James. 2nd ed. Boca Raton, FL: CRC Press. 423-436.
Zuk, M., & McKean, K.A. 1996. Sex differences in parasite infections: Patterns and processes. *International Journal for Parasitology* 26 (10):1009-1024.

10. Appendices

10.1 Appendices relevant to Chapter 4

10.1.1 *Preliminary screening and isolation*

10.1.1.1 Xylose Lysine Deoxycholate agar plates

Xylose Lysine Deoxycholate Agar (XLD) (BD Diagnostics, Maryland, USA) was prepared according to the manufacturer's recommendations and used as a selective and differentiation media for the growth and isolation of *Salmonella*. Briefly, 55 g of the dehydrated media were dissolved in 1 L of distilled water (DW) and mixed thoroughly. The solution was then heated until boiling point. It was then left until it reached 55 – 60 ˚C and used immediately to prepare the culture plates. Plates were then incubated at 37 ˚C for 24 h for quality control. XLD agar plates contain xylose, which is fermented by practically all Enterobacteriaceae except *Shigella* species. It also contains lysine that enables *Salmonella* to be differentiated from the non-pathogenic Enterobacteriaceae by slowing down the fermentation process of *Salmonella* on the xylose. Additionally, it contains an indicator system that allows the visualisation of hydrogen sulphide (H_2S) produced by *Salmonella* colonies, resulting in the formation of colonies with black centres, while sodium deoxycholate is use to inhibit gram-positive organisms (Zimbro *et al.* 2009). Typical *Salmonella* colonies in XLD, appear either red/pink (although the colony in itself is colourless) with or without pale/dark black centres (Wallace, Jacobson, & Hammack 2009). If typical colonies were not present, atypical *Salmonella* colonies (i.e. light yellow or yellow colonies with or without black centres) were then examined (Wallace, Jacobson, & Hammack 2009).

10.1.1.2 Direct method with pre-enrichment

This method was used to subjectively estimate the load of *Salmonella* in the sample. It involved a pre-enrichment step with buffered peptone water (PW) used to resuscitate *Salmonella* that had been damaged due to conditions such as prolonged storage or freezing-thawing (Davies 2013). Briefly, 0.5 g of the inner most part of the faecal sample were mixed with 5 mL of sterile PW in a 10 mL polycarbonate yellow cap sterile tube (SARSTEDT Aktiengeseilschaft & Co. Germany) and incubated at 37 ˚C for 20 h in a shaker. A 1:10 w/v ratio was used, as this same ratio was required in the indirect method with enriching medium. Following incubation, a sterile cotton tip applicator (Multigate Medical

Products PTY LTD., New South Whales, Australia) was used to inoculate single XLD plates with a heavy inoculum using the entire surface of the agar plate; plates were labelled, sealed with Parafilm® (BEMIS, Wisconsin, USA) and incubated at 37 ˚C for at least 24 h. A positive culture plate had bacterial growth compatible with *Salmonella* (see below).

10.1.1.3 Indirect method with Delayed Secondary Enrichment (DSE)

This method was used for the isolation and further identification of *Salmonella*. In addition to a pre-enrichment step (that of the indirect method), it also involved a selective enrichment step with Rappaport Vassiliadis (RV) broth (Oxoid LTD., Hampshire, England) that enhances the growth of *Salmonella* while inhibiting the growth of other organisms, therefore increasing the likelihood of subsequent isolation. Rappaport Vassiliadis was prepared according to laboratory instructions. Briefly, 30 g of the dehydrated media were mixed in 1 L of distilled water while gently heated on a magnetic stirrer hot plate thermostat (IEC Pty Ltd, Australia) until dissolved. Upon preparation of the media, 10 mL of this was dispensed in sterile single 50 mL polycarbonate screw-capped McCartney bottles (TechnoPlas Pty Ltd, St. Marys, South Australia) and autoclaved at 115 ˚C for 15 min, after which vials were stored at 4 ˚C for further use. Rappaport-Vassiliadis enrichment broth vials were inoculated with 10 µL of the already inoculated PW that was originally prepared for the direct method previously mentioned, and incubated at 42 ± 1˚C for 48 hours in a water bath. A single un-inoculated RV vial was also incubated as a negative control. A positive growth on RV enrichment broth was considered to be that in which cloudiness of the media was present. Inoculation of XLD plates was performed by placing 30 µL of the inoculated RV enrichment broth on the surface of the agar and streaking out for single colonies with a sterile 10 µL disposable inoculation loop (SARSTEDT Aktiengeseilschaft & Co. Germany). Plates were sealed with Parafilm® (BEMIS, Neenah, USA) and incubated for 18-24 hours at 37 ˚C. Assessment of morphology and growth was done as previously described for cultures on XLD with the direct method.

10.1.2 ***Preliminary identification and storage***

10.1.2.1 Gram stain protocol

Bacterial smears were prepared from colonies in nutrient agar (NA). Fresh microscope slides were cleaned with Kimwipes Kimtech® (Kimberly-Clark Worldwide Inc., Roswell, USA) and flamed. A small amount of a bacterial colony was emulsified in a drop of distilled water, and the smear was then fixed by heat. Smears were then Gram stained using a modified protocol of that by Markey et al. (2013). Briefly, crystal violet was applied for 30

sec followed by Gram's iodine (fixative) for 30 sec as well. Then Gram's decolouriser (acetone) was added for 10 sec, upon which carbol fuchsin (counter-stain) was applied for 30 sec. Water washes were applied in between.

10.1.2.2 Biochemical tests

Initially, a PW broth was inoculated with a single colony from an NA plate. Subsequently, 3 – 5 drops of this inoculated PW were used to inoculate all other test tubes using a 3 mL sterile polyethylene disposable transfer pipette (SARSTEDT Aktiengeseilschaft & Co. Germany) and incubated at 37˚C for 24-48h with the lids loose. Table 10-1 shows the tests performed and their corresponding description.

Table 10-1 Biochemical tests used for the preliminary identification of suspicious isolates on XLD agar plates

Test	Description and protocol
Indole (I)	determines the ability of an organism to produce indole from tryptophan. Briefly, 0.5mL of Kovac's reagent were added to the PW tube, gently shaken and examined after 2 mins. Indole positive was indicated as a red colour in the reagent layer, while an indole negative was indicated by a yellow colour in the reagent layer.
Methyl Red test (MR)	determines the ability of an organism to produce and maintain stable acid end products from the fermentation of glucose. Briefly, two drops of methyl red solution (School of Veterinary and Biomedical Sciences, Murdoch University) were added to the MR tube test, shaken gently and examined immediately. A methyl red positive test was considered to be a strong red colour, and a methyl red negative test was that one with a yellow colour
Voges-Proskauer test (VP)	determines the ability of an organism to convert the acid end products of glucose fermentation to neutral end products. This test was carried out by transferring 250 µL of MR culture media to a 1.5mL PCR tube (SARSTEDT Aktiengeseilschaft & Co. Germany), then 150 µL of solution VP A (5% α-naphthol in ethanol) and 50 µL of solution VP B (40% KOH) were added, the tube was gently shaken and the reaction was read 10-20 min later. A test was considered to be VP positive if the reaction colour was red and negative if colourless
Citrate (C)	determines the capacity of an organism to use citrate as a sole source of carbon. Briefly, the slant citrate media was inoculated with three to five drops of inoculated PW broth with a 3 mL sterile polyethylene disposable transfer pipette (SARSTEDT Aktiengeseilschaft & Co. Germany), and the tube was then gently rotated to make sure the PW broth covered the entire surface of the citrate agar. A citrate positive test is one in which the media has changed to blue and has visible growth, where as a negative citrate test does not have any growth and there is no colour change
Urea hydrolysis (U)	determines the organism's ability to hydrolyse urea to ammonia. A positive urease test its indicated by a bright pink colour in the urea medium, where as a negative urease test is indicated by no colour change
Lactose (carbohydrate fermentation) (L)	determines the ability of the organism to ferment or not ferment lactose as a carbon energy source. A positive lactose test is indicated by a change in colour from red/pink to yellow while a negative lactose test has no colour change post incubation
Lysine decarboxylase (LD)	determines the presence and activity of lysine decarboxylase which decarboxylates lysine to produce amine 1,5-diaminopentane or cadaverine. With acid production, due to glucose fermentation, the medium first turns yellow. Then, if decarboxylation of lysine occurs, the medium then turns violet in response to the production of alkaline end-products. This test is performed with a blank control tube that doesn't contain lysine. Both tubes are inoculated with the PW broth media and a layer of sterile Parafilm oil is added as exposure to air could cause alkalinisation of the media resulting in a false positive reaction. A positive lysine decarboxylase reaction is indicated by a purple/violet colour with turbidity, where as a negative lysine decarboxylase reaction is indicated by a yellow colour or no change without turbidity

10.1.2.3 Antiserum agglutination

This test represented the last step in the preliminary identification of suspicious *Salmonella* isolates before storing for further processing. Antiserum Salmonella Omnivalent Omni-O (A-60) and Antiserum Salmonella Polyvalent OMG (both from Bio-Rad laboratories, Marnes-la-Coquette, France), were used for this purpose. The procedure required pure fresh colonies from NA plates. Briefly: one drop of sterile water (Water for Injection BP, AstraZeneca Pty Ltd., North Ryde, Australia) and one drop of antiserum were placed separately on a microscope slide (Waldemar Knittel, Germany). Using separate sterile 10µL disposable inoculation loops (SARSTEDT Aktiengeseilschaft & Co. Germany), one colony was mixed with the drop of distilled water to be used as a negative control and one colony mixed with the antiserum drop as well. Colonies were suspended making sure a homogeneous mixture of both drops was obtained. The slide was then rocked gently in a rotary movement. A positive reaction was one in which there was agglutination with the antiserum; a reaction within the first 30 sec was considered strong, and a reaction in between 30 and 60 sec was considered weak. A valid positive reaction was one in which agglutination was not observed on the negative control. If agglutination was observed on the control suspension, the isolate was stored and sent for further identification, as all other tests were compatible with *Salmonella*.

10.1.2.4 Storage

Suspicious and confirmed *Salmonella* isolates were subsequently stored in Protect® Bacterial Preservers (Technical Service Consultants Limited, Lancashire, United Kingdom) cryovials according to the manufacturer's guidelines. Briefly, a 10 µL sterile disposable inoculation loop (SARSTEDT Aktiengeseilschaft & Co. Germany) was used to harvest a loop full of single colonies from the NA plate from which all previous tests had been performed. The Protect® vial was then open, flamed and the colonies were inoculated onto the cryopreservative fluid and homogenised until a thick suspension was obtained. Subsequently, the vial was then flamed, capped and gently inverted six times and left to stand for at least 30 sec, after which most of the cryopreservative fluid was withdrew using a 3 mL sterile polyethylene disposable transfer pipette (SARSTEDT Aktiengeseilschaft & Co. Germany), having flamed the vial just previously. Lastly, the vial was flamed, recapped and stored at -80 ˚C for further processing.

10.1.3 ***Revival, serotyping and antibiotic sensitivity***

Cryovials were transferred from -80 ˚C to a cryoblock (Technical Service Consultants Ltd. Lancashire, England), which had been pre-cooled to -20 ˚C for at least 1 h to extend the

available working time with the isolates. In a laminar flow hood, the storage vial was opened, flamed and a single bead was extracted using a sterile hypodermic needle (Becton Dickinson, Franklyn Lakes, USA). Subsequently, the vial was flamed, capped and returned to the cryoblock. The bead was placed in nutrient broth (NB) media and incubated at 37 ˚C for 24 h. Having confirmed growth by turbidity in the medium, 30 µL of the suspension were inoculated on separate plates of NA and XLD and streaked out for single colonies. Plates were incubated at 37 ˚C for 24 – 48 h. Colony morphology on XLD was used to confirm that the revived organism had *Salmonella* morphology (quality control) and therefore confirming the growth on NA. The 24 – 48 h range was used to allow the bacteria to reactivate and present its characteristic morphology. Inspection for growth and contamination was done twice daily. If colony morphology (on XLD) corresponded to the one in the records for that given isolate, a single colony on the NA plate was then subcultured onto a slant NA media prepared in 50 mL polycarbonate McCartney bottles previously autoclaved (TechnoPlas Pty Ltd, St. Marys, South Australia) and incubated at 37˚C for 18 – 24 h. Isolates were then submitted to the national reference laboratory for *Salmonella* in Perth at PathWest, Sir Charles Gairdner Hospital, Western Australia for serotyping by antisera slide agglutination (Kauffmann-White-LeMinor scheme) to detect O (somatic), H (flagellar) and K (capsular) antigens, which today is still widely accepted as the gold standard for identification of *Salmonella* isolates. Two isolates were sent to the Australian Salmonella Reference Centre (Institute of Medical and Veterinary Science; IMVS Pathology) for further serotyping. Upon serovar identification, isolates were revived once more as previously described. A colony was subsequently subcultured onto a NA plate, streaked out for single colonies and incubated at 37˚C for 18 – 24 h. Colonies in this plate were subsequently used to perform the antimicrobial susceptibility test, using the disk diffusion susceptibility method (gold standard) (Bauer *et al.* 1966). Briefly, a bacterial inoculum of approximately 1-2 x 10^8 CFU/mL was applied onto a 150 mm (diameter) Mueller-Hinton agar (MHA) plate in a lawn format. Paper antibiotic disks with a fixed concentration of the drug were deployed on the surface of the MHA plate and incubated for 16-24 h at 35 ˚C. Susceptibilities of the isolates, which provide qualitative results (i.e. sensitive, intermediate, or resistant), were then obtained by measuring the zones of growth inhibition around each disk, which were then compared against the criteria of the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute 2013).

10.2 Appendices relevant to Chapter 5

10.2.1 *Isolation and storage protocols*

Inoculation of the BSA plates was by wiping the swabs over the entire area of the agar. Plates were labelled and sealed with Parafilm® (BEMIS, Wisconsin, USA) paper and incubated at 28±0.5 ˚C for 4 weeks. Daily inspection optimised identification of colonies with yeast-suspicious morphology, i.e. opaque, mucoid in consistency (evident as smooth, high light reflectance), convex or flat in elevation, mostly circular (although varying shape), usually with an amorphous (i.e. uniform) structure, an entire edge, and produce yeast fermenting odour. Plates were discarded if contaminated with filamentous fungi or bacteria.

Suspicious colonies with BCE were then subcultured onto fresh BSA plates to further confirm the BCE in the absence of other growth. Suspicious colonies were subsequently subcultured onto Sabouraud dextrose agar (SDA) plates (Microbiology, School of Veterinary & Biomedical Sciences, Murdoch University). Two SDA plates were inoculated and streaked out for single colonies. One plate was incubated at 28 ˚C; the second was incubated at 37±0.5 ˚C to confirm growth at animal body temperature. Both were checked daily for growth and contamination. Preliminary confirmation of *Cryptococcus* spp. like yeast organisms was done through a series of tests on colonies from SDA incubated at 28±0.5 ˚C.

A Gram stain was performed by emulsifying a small colony in 15 µL of sterile water on a microscope slide (Waldemar Knittel, Germany) and fixed by heat. The slides were then Gram-stained and observed under an Olympus BX50F4 microscope (Olympus Optical Co, Ltd. Japan) at 100x magnification. Typical ovoid or circular Gram-positive cells, reproducing by budding, were considered confirmation of yeast.

An India ink capsule positive test (Scientific Device Laboratory, Des Plaines, USA) was then used to visualise the capsule (characteristic of *Cryptococcus* species as well as some other yeast such as *Rhodotorula* spp.). A small portion of a colony or a very small colony was emulsified on a microscope slide (Waldemar Knittel, Germany) in a drop of a 1:1 solution of sterile water (Water for Injections BP, AstraZeneca Pty Ltd., North Ryde, Australia) and India ink. The slides were coverslipped and observed under an Olympus BX50F4 microscope (Olympus Optical Co, Ltd. Japan) at 100x magnification. The presence of a

capsule was confirmed by a light halo around the cell (caused by India Ink particles being exclude from the cell). Subsequently, Gram-positive, budding-cell colonies scored as capsule-positive were then tested by the Christensen's Urea Agar test (CUAT). This test is the standard biochemical method for urease activity detection (Canteros *et al.* 1996 ; Christensen 1946). *Cryptococcus* spp. have the ability to hydrolyse urea and generate a pH change; results of this test can therefore be used for the preliminary differentiation from other yeasts. A heavy inoculum (i.e. not less than 10 colonies) was harvested usually from a 3 day-old SDA culture and plated onto slant Urea Base Agar (Oxoid Laboratories) and incubated at 28±0.5 ˚C and checked daily for growth and colour change of the media. As a negative control, an uninoculated CUAT was always included. A positive urease reaction was indicated by the agar turning pink.

Lastly, colonies with yeast suspicious morphology on culture media, that were Gram-positive (oval or circular) with evidence of reproduction by budding, capsule positive under India ink technique, and urease positive, were subcultured one last time onto SDA plates and incubated at 28±0.5 ˚C. Colonies from SDA pure cultures were stored in Microbank™ System (PRO-LAB Diagnostics, Richmond Hill, Canada) cryovials according to the manufacturer's guidelines. Briefly: a loop (10 µL sterile disposable inoculation loop, SARSTEDT Aktiengeseilschaft & Co. Germany) full of single colonies[16] was used to inoculate a Microbank™ cryovial that had been previously flamed. Colonies were mixed until completely emulsified. Subsequently, the vial was flamed once more, and the cap put back. The vial was then mixed by inversion five times. The excess cryopreservative was aspirated out of the vial using a 3 mL sterile disposable transfer pipette (SARSTEDT Aktiengeseilschaft & Co. Germany) having flamed the vial previously. The vial was then stored at -80 ˚C for further processing.

10.2.2 *Revival and preliminary identification protocols*

Cryovials were transferred from -80 ˚C to a cryoblock (Technical Service Consultants Ltd. Lancashire, England), which had been pre-cooled to -20 ˚C for at least 1 h to extend the available working time with the isolates. In a laminar flow hood, the storage vial was opened, flamed and a single bead was extracted using a sterile hypodermic needle (Becton Dickinson, Franklyn Lakes, USA). Subsequently, the vial was flamed, capped and returned to the cryoblock. The bead was placed directly onto an SDA plate and moved on its surface as if streaking out. Lastly, culture plates were labelled, sealed with Parafilm® (BEMIS,

[16] due to the slow growth rate of the isolated organisms, colonies older than 24 h were stored in Microbank™ cryovials and not 18-24 h as indicated by the manufacturer

Wisconsin, USA) and incubated at 28±0.5 ˚C. for at least 3-5 days before growth started to appear.

For preliminary biochemical identification, the API® 20 C AUX identification system for yeast (bioMérieux SA, Marcy-I'Etoile, France) was used according to the manufacturer's guidelines. Briefly: In-house 0.85% sodium chloride (NaCl) was prepared in aliquots of 2 mL in 5 mL sterile Macartney Bottles and autoclaved at 121˚C for 15 min, for the inoculation of the API 20C AUX strips and kept at room temperature. When inoculating the API® 20 C AUX strips, the incubation box (tray and lid) was prepared by adding 5 mL of sterile water (Water for Injections BP, AstraZeneca Pty Ltd., North Ryde, Australia) onto the tray, making sure it was evenly distributed. Subsequently, one API® 20 C AUZ strip was removed from its packaging and delicately place into the tray, making sure no water would get in contact with the culture cupules. Colonies were then harvested from pure culture SDA plates using a 10 µL sterile disposable inoculation loop (SARSTEDT Aktiengeseilschaft & Co. Germany) and mixed thoroughly with 2 mL of 0.85% NaCl until a suspension with a turbidity equal to 2 McFarland was obtained. Next, an ampule of API C medium was inoculated with 100 µL of the NaCl 0.85% suspension using a 3 mL sterile polyethylene disposable transfer pipette (SARSTEDT Aktiengeseilschaft & Co. Germany) by gentle suction (taking care to not form bubbles). The strip cupules (with the multiple culture media tests) except one (control) where then inoculated with 5 drops of the previously prepared suspension, taking care of not forming bubbles and to not overfilling the cupules. The tray was then covered with its lid and incubated at 28±0.5 ˚C (temperature at which isolates had been cultured previously). Readings were done at 48 and 72 h. A positive reading was that of a cupule with grater turbidity than the control cupule. Interpretation of the numerical profile obtained (according to the result of each cupule) was done using the apiweb™ identification software with database v4.0 (bioMérieux SA, Marcy-I'Etoile, France).

10.2.3 *DNA extraction and molecular identification protocols*

The PowerSoil™ DNA isolation protocol was modified by adding four freeze-thaw steps, each time using liquid nitrogen and boiling water (10 min), and a step with protein kinase lysis over night. More specifically, a loop full of fresh pure culture colonies of a 10 µL sterile disposable inoculation loop (SARSTEDT Aktiengeseilschaft & Co. Germany) were inoculated onto the PowerBead Tubes provided, that were then gently vortexed. Subsequently, four steps of freeze-thaw in liquid nitrogen and boiling water were performed with a final thawing step of 5 min. Solution C1 was then added (60 µL) and the

PowerBead Tubes were vortexed briefly initially and then for 10 min at maximum speed. Tubes were then centrifuged at 10,000 x g for 30 sec at room temperature, after which the supernatant was transferred to a clean 2 mL Eppendorf tube. Subsequently 250 µL of solution C2 were added to the tubes and vortexed for 5 seconds. Proteinase K (i.e. 25 µL) was then added to each tube and incubated on a hot plate/shaker at 56 ˚C and 650rpm. Tubes were then centrifuged at room temperature for 1 min at 10,000 x g. Avoiding the pellet, up to 600 µL of supernatant was transferred to a clean 2 mL Eppendorf tube (SARSTEDT Aktiengeseilschaft & Co. Germany). Solution C3 was added (i.e. 200 µL) to each tube and vortexed briefly and then left to incubate at 4 ˚C for 5 min. Tubes were then centrifuged at room temperature for 1 min at 10,000 x g. Avoiding the pellet formed, 750 µL of the resultant supernatant was transferred into a clean 2 mL Eppendorf tube, then solution C4 was added (750 µL) and the tube was vortexed for 5 sec. At this stage, three loads of 675 µL of the resulting solution were added onto a Spin Filter separately and centrifuged at 10,000 x g for 1 min at room temperature (the flow through gets discarded after every load). Having discarded the flow through after the third load and spin, 500 µL of solution C5 were loaded onto the Spin Filter and centrifuged at room temperature for 30 sec at 10,000 x g. The resulting flow through gets discarded and the Spin Filter was centrifuged one more time for 1 min at 10,000 x g. Subsequently the Spin Filter was placed in a new clean 2 mL Eppendorf (Sarstedt, Numbrecht, Germany), and DNA was eluded by adding 100 µL of solution C6 directly onto the filter membrane and centrifuged at room temperature for 30 sec at 10,000 x g. DNA was then stored at -20 ˚C for downstream applications.

Extracted DNA from isolated yeast organisms was tested with Polymerase Chain Reaction (PCR). Primers ITS1 (forward) 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4 (reverse) 5'-TCCTCCGCTTATTGATATGC-3' were used to amplify the Internal Transcribed Spacer (ITS) region in rDNA from all fungal isolates with an expected amplicon that varies among species (e.g. 710 bp in *Saccharomyces cerevisiae* and *Suillus sinuspaulianus*; in *Rhizopogon* spp. the region could be up to 850 bp) but is usually bigger than 700 bp. These primers make use of conserved regions of the 18S, 5.8S, and 28S rRNA genes to amplify the noncoding regions (i.e. ITS 1 and ITS 2) between them (White *et al.* 1990) (Figure 10-1).

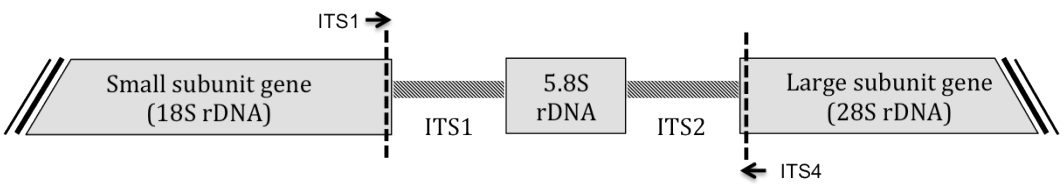


Figure 10-1 Three coding and two internal transcribe spacer regions of the fungal nuclear ribosomal DNA, with approximate locations of PCR forward primer ITS1 and reverse primer ITS4 (dotted lines) used in this

study. The arrows represent the 3' end of each primer.

PCR reactions were carried out in 25 µL reaction volumes containing 1 µL of DNA template, a working concentration of 0.5 µM of each primer (i.e. upstream: ITS1, and downstream: ITS4), and 2x PCR Master Mix (Promega Corporation, Madison, USA) containing 50 units/mL Taq DNA polymerase, 1.5 mM of $MgCl_2$, and 200 µM of dNTPs. Cycling conditions were as follow: an initial denaturation of 1 min at 95˚C; 40 cycles with 95˚C for 30 sec, 50˚C for 45 sec, and 72˚C for 1 min; and one last cycle with 95˚C for 30 sec, 50˚C for 45 sec, and 72˚C for 7 min. PCR products were visualised in 2% agarose gels, ran at 90 V, 400 mAmp for 45 min. Bands were then cut out with separate sterile scalpel blades, and DNA was then purified using the filter tip method. Briefly, 100 µL filter tips were previously prepared by cutting enough off the bottom so they could fit into a 1.5 mL Eppendorf tube (previously UV sterilised) with the lid closed. Excess agarose gel was trimmed off the cut bands and subsequently placed into the filter tips (already inside the Eppendorf tubes). At this stage, Eppendorf tubes were centrifuged at 16,100 x g for 2 min upon which the filter tip was removed. Agarose gel was removed by the filter membrane technique and the resulting flow through contained the purified PCR reaction product. All PCR and sequencing reactions were carried out in an Applied Biosystems (AB) GeneAmp 2720 Thermal Cycler.

Sequencing was done at a 1/8 reaction using 1 µL of dye terminator mixture (Applied Biosystems, Scoresby, Australia) and 1.5 µL of 5x sequencing buffer (SABC, Murdoch University) in a 10 µL final reaction volume (a full reaction uses 8 µL of dye terminator in 20 µL total volume) given that the concentration of DNA post PCR was within the expected threshold of 5-20 ng (DNA template required for a half reaction, that is 10 µL final reaction volume) as recommended by Applied Biosystems in their BigDye® Terminator v3.1 Cycle Sequencing Kit protocol. Briefly, a master mix containing 1 µL of dye terminator mixture, 1.5 µL of 5x sequencing buffer, 1 µL of 3.2 pM working concentration primer (ITS4), and 4.5 µL of PCR grade water (Fisher Biotech, Perth, Australia) was prepared for each PCR product to be sequenced. Lastly, 2 µL of purified DNA (i.e. cut band) were added to each reaction. Tubes were then gently vortexed and centrifuged for less than 5 sec to ensure reactions were well mixed and at the bottom of the tubes. Cycle conditions were: an initial step of 96˚C for 2 min (samples were placed on the plate when the temperature was near 96˚C), then 25 cycles of 96˚C for 10 sec (denaturation), 50˚C (annealing temperature for the ITS4 primer used in the initial PCR protocol) for 5 sec, then 60˚C for 4 min (extension), and lastly a indefinite holding step at 14˚C.

Purification of sequence reaction products was done using the ethanol precipitation protocol for BigDye® Terminator v3.1 (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Scoresby, Australia) method. For each reaction, the entire sequencing reaction product was transferred to a 0.5 mL Eppendorf tube previously UV sterilised. Subsequently, in the following order, 1 µL of 125mM EDTA, 1 µL of 3M Sodium acetate pH 5.2 and 25 µL of 100% Ethanol were added to each reaction tube. Tubes were then briefly vortexed at low speed and left to incubate at room temperature for at least 20 min after which, tubes were centrifuged at maximum speed for 30 min. At this stage, the resulting supernatant was removed using a vacuum pump with P200 filter tips (previously UV sterilised) and tubes were left upside down to dry for at least 3 min. The DNA pellet (not visible) was then gently rinsed with 125 µL of 70% Ethanol, followed by a microfuge step at maximum speed for 5 min at room temperature. Lastly, all the supernatant was removed using a vacuum pump with P200 filter tips (previously UV sterilised). Tubes were placed in a Speedvac Concentrator (Savant Instruments, Inc. New York) to dry out and spin for 15 min before being submitted to the sequencing facility at the State Agricultural and Biotechnology Centre (SABC) located in Murdoch University, Western Australia.

10.3 Appendices relevant to Chapter 6

10.3.1 *DNA extraction from peripheral whole blood*

The spin-column protocol for whole blood of the QIAamp® DNA MIni Kit (QIAGEN®, Hilden, Germany) was used. Briefly, 20 µL of Proteinase K initially added to individual 1.5 mL microcentrifuge tubes. Subsequently, the maximum volume possible of whole anticoagulated blood (ranged from 25 µL to 100µL) was added to the microcentrifuge tubes and mixed with the proteinase K by pipetting up and down a couple of times. The total volume was then adjusted to 220 µL by adding phosphate buffered saline (PBS) and mixed by pipetting up and down. Then, 200 µL of buffered AL were added to the sample and microcentrifuge tubes were thoroughly mixed by pulse-vortexing for 15 s. Tubes were then incubated on a hot plate/shaker at ~56 ˚C and 650 rpm for 10 min, upon which the mixture was then briefly centrifuged. Next, 100% ethanol (200 µL) was added to each sample and mixed thoroughly by pulse-vortexing for 15 s. and then centrifuged once more. The suspension was then carefully transferred to a QIAamp Mini spin column, closed and centrifuged at 6,000 x g for 1 min. Upon centrifugation, the filtrate was discarded and the spin column was put into a new 2 mL Eppendorf tube. Buffered AW1 was then added (500 µL) to the spin column and subsequently centrifuged at 6,000 x g for 1 min. Once again the spin column was transferred to a new clean 2 mL Eppendorf tube and the filtrate was discarded. With the spin column in a new 2 mL Eppendorf tube, buffered AW2 was added (500 µL) and centrifuge at 20,000 x g for 3 min. Next, the spin column was transferred once more to a clean 1.5 mL Eppendorf tube and the filtrate discarded. Lastly, the elution step was done by adding 200 µL of buffer AE onto the spin column, which was then incubated at room temperature (15-25 ˚C) for 1 min, and then centrifuge at 8,000 rpm for 1 min.

Extraction of DNA from whole blood with ISOLATE II Blood DNA Kit (Bioline Reagents Ltd., London, United Kingdom) involved a lysing step in a 1.5 mL microcentrifuge tube with 25 µL of Proteinase K mixed with whole blood in volumes ranging from 25 µL to 100 µL. Adding PBS then adjusted this to a total volume of 200 µL. Buffer G3 (200 µL) was added to the homogenate, which then was mixed by vortexing for 20 sec. Microcentrifuge tubes were then incubated on a hot plate/shaker at ~70 ˚C and 650 rpm for 30 min. DNA binding conditions were then adjusted by adding 210 µL of 100% ethanol and vortexed. Subsequently, each homogenate was then placed into an ISOLATE II Blood DNA Spin Column (within a 2 mL collection microcentrifuge tube) and centrifuged at 11,000 x g for 1 min. Samples that did not filtered entirely, were centrifuged once more at a higher g force

until no sample was left above the silica membrane. The Spin Column was then washed with buffer GW1 (500 µL) and centrifuged for 1 min at 11,000 x g. The Spin Column was then transferred to a new collection tube and the filtrate discarded. Buffer GW2 was added next (600 µL) to the spin column and centrifuged for 1 min at 11,000 x g, and the filtrate discarded. Residual ethanol was removed by a last centrifugation step of the spin column at 11,000 x g for 1 min. The spin column was then transferred to a new clean 1.5 mL Eppendorf tube and 100 µL of elution buffer G (preheated at 70 ˚C) were added onto the silica membrane and left incubating at room temperature (15-25 ˚C) for 1 min, before a last centrifugation step at 11,000 x g for 1 min.

Next, purity and quantity of the DNA extracted from paired samples using QIAGEN and Bioline kits were compared using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific™, Waltham, USA). The ratio of absorbance at 260 nm and 280 nm (260/280) as well as the concentration of DNA/RNA recovered per µL were similar for both methodologies. All DNA products were then tested by PCR, and reactions were then visualized in agarose gel (see protocols below). Since all samples were negative, a spike analysis was then carried out to rule out the presence of potential inhibitors in the DNA extracted with both the QIAGEN and the Bioline kits. For this, all PCR reactions were loaded with 0,5 µL of positive control template (equine herpesvirus type 1 or EHV-1). Subsequent results were satisfactory, as all PCR reactions provided a band on the agarose gel of the same size as the positive control. Sequenced data obtained from one of the bands confirmed that EHV-1 was amplified. Consequently, the remaining blood samples were therefore extracted using ISOLATE II Blood DNA Kit (Bioline Reagents Ltd., London, United Kingdom).

10.3.2 *Nested PCR and sequencing methods*

This process followed previously described protocols (VanDevanter *et al.* 1996). Briefly, primary reactions contained two forward primers: DFA (5'- GAYTTYGCNAGYYTNTAYCC - 3') and ILK (5'- TCCTGGACAAGCAGCARNYSGCNMTNAA - 3'); and one reversed primer KG1 (5' - GTCTTGCTCACCAGNTCNACNCCYTT - 3'). Secondary reactions were run with 0.5 µL of the primary reaction product, with one forward primer TGV (5' - TGTAACTCCGGTGTAYGGNTTYACNGGNGT - 3') and a reverse primer IYG (5' - CACAGAGTCCGTRTCNCCRTADAT - 3'). These primers were used to amplify a highly conserved region of the herpesviral DNA-dependant-DNA polymerase (DPOL) gene, with an expected sequence of 215 to 315 bp. PCR reactions were carried out both primary and secondary, in 25 µL reaction volumes containing 1 µL of DNA template (primary reaction)

and 0.5 µL of the primary reaction product for the secondary reaction, a working concentration of 0.5 µM of each primer and 2X GoTaq® Hot Start Green Master Mix (Promega Corporation, Madison, USA) in reaction buffer (pH 8.5), 4 mM of $MgCl_2$, and 400 µM of each dNTPs. Cycling conditions were the same for both, the primary and secondary reactions: an initial denaturation of 2 min at 94 ˚C; 45 cycles with 94 ˚C for 30 s (denaturation), 46 ˚C for 60 s (annealing), and 72˚C for 60 s (extension); and a final extension with 72 ˚C for 7 min. All PCR and sequencing reactions were carried out in an Applied Biosystems (AB) GeneAmp 2720 Thermal Cycler.

PCR products were visualised in 1.5% agarose gels made of 0.5x TBE buffer, stained with SYBR-Safe (Life Technologies, Carlsbad, USA) and ran at 90 V, 400 mAmp for ~45 min. Bands of the expected size were cut out using separate sterile scalpel blades and DNA was purified from agarose suing the filter tip method. Briefly, 100 µL filter tips were previously prepared by cutting off the bottom enough so they could fit into a 1.5 mL microcentrifuge tube (previously UV sterilised) with the lid closed. Excess agarose gel was trimmed off the cut bands and subsequently placed into the filter tips (already inside the Eppendorf tubes). At this stage, microcentrifuge tubes were centrifuged at 15,000 rpm for 2 min upon which the filter tip was removed. The filter membrane removed the agarose gel and the resulting flow through contained the purified PCR reaction product.

10.3.2.1 Sequencing

Sequencing was done at a 1/8 reaction using 1 µL of dye terminator mixture (SABC, Murdoch University) and 1,5 µL of 5x sequencing buffer (SABC, Murdoch University) in a 10 µL final reaction volume (a full reaction uses 8 µL of dye terminator in a 20 µL reaction) given that the concentration of DNA post PCR was within the expected threshold of 5-20 ng (DNA template required for a half reaction, that is 10 µL final reaction volume) as recommended by Applied Biosystems in their BigDye® Terminator v3.1 Cycle Sequencing Kit protocol. Briefly, a master mix containing 1 µL of dye terminator mixture, 1.5 µL of 5x sequencing buffer, 1 µL of 3.2 pmoles working concentration primer (TGV forward primer), and 4.5 µL of PCR grade water (Fisher Biotech, Perth, Australia) was prepared for each PCR product to be sequenced. Lastly, between 2-3 µL of purified DNA (i.e. cut band) were added to each reaction. Tubes were then gently vortexed and centrifuge for less than ~5 seconds to ensure reactions were well mixed and at the bottom of the tubes. Cycle conditions were: an initial step of 96 ˚C for 2 m (samples were placed on the plate when the temperature was near 96 ˚C), then 25 cycles of 96 ˚C for 10 s (denaturation), 46 ˚C (annealing temperature of the amplification protocol) for 5 s, then 60 ˚C for 4 m

(extension), and lastly an indefinite holding step at 12 ˚C. PCR products were sequenced in both directions (forward and reverse).

Purification of sequence reaction products was done using the ethanol precipitation protocol for BigDye® Terminator v3.1 (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Scoresby, Australia) method. For each reaction, the entire sequencing reaction product was transferred to a 0.5 mL microcentrifuge tube previously UV sterilised. Subsequently, in the following order 1 µL of 125mM EDTA, 1 µL of 3M Sodium acetate pH 5.2 and 25 µL of 100% ethanol were added to each reaction tube. Tubes were then briefly vortexed at low speed and left to incubate at room temperature for at least ~20 m upon which tubes were centrifuged at maximum speed for 30 min. At this stage, the resulting supernatant was removed using a vacuum pump with P200 filter tips (previously UV sterilised) and tubes were left upside down to dry for at least 3 m. The DNA pellet (not visible) was then gently rinsed with 125 µL of 70% Ethanol, followed by a microfuge step at maximum speed for 5 m at room temperature. Lastly, all supernatant was removed using a vacuum pump with P200 filter tips (previously UV sterilised). Tubes were placed in a Speedvac Concentrator (Savant Instruments, Inc. New York) to dry out and spined for ~15 m before being submitted to the sequencing facility at the State Agricultural and Biotechnology Centre (SABC) located in Murdoch University, Western Australia. Sequencing was carried out on a ABI 3790 96 capillary automatic sequencer (Applied Biosystems, Scoresby, Australia).

10.4 Appendices relevant to Chapter 7

10.4.1 *DNA extraction from peripheral whole blood*

Extraction of DNA from whole blood with ISOLATE II Blood DNA Kit (Bioline Reagents Ltd., London, United Kingdom) involved a lysis step in a 1.5 mL microcentrifuge tube with 25 µL of Proteinase K mixed with whole blood in volumes ranging from 25 µL to 100 µL. Adding Phosphate-buffered saline (PBS) then adjusted this to a total volume of 200 µL. Buffer G3 (200 µL) was added to the homogenate, which then was mixed by vortexing for 20 s. Microcentrifuge tubes were then incubated on a hot plate/shaker at 70 ˚C and 650rpm for 30 min. DNA binding conditions were then adjusted by adding 210 µL of 100% ethanol and vortexed. Subsequently, each homogenate was then placed into an ISOLATE II Blood DNA Spin Column (within a 2 mL collection microcentrifuge tube) and centrifuged at 11,000 x g for 1 min. Samples that did not filter completely were centrifuged once more at a higher g-force until no sample was left above the silica membrane. The Spin Column was then washed with buffer GW1 (500 µL) and centrifuged for 1 min at 11,000 x g. The Spin Column was then transferred to a new collection tube and the filtrate discarded. Buffer GW2 was added next (600 µL) to the spin column and centrifuged for 1 min at 11,000 x g, and the filtrate discarded. Residual ethanol was removed by a last centrifugation step of the spin column at 11,000 x g for 1 min. The spin column was then transferred to a new clean 1.5 mL Eppendorf tube and 100 µL of elution buffer G (preheated to 70 ˚C) was added onto the silica membrane and left to incubate at room temperature for 1 min, before a final centrifugation step at 11,000 x g for 1 min. Extracted DNA was stored at -4 ˚C.

10.4.2 *PCR amplification*

10.4.2.1 Piroplasms

Samples were screened for piroplasms, using a nested-PCR method that uses universal primers to amplify a 850 bp fragment of the 18S ribosomal RNA gene (18S rDNA) of piroplasms, as previously described (Jefferies, Ryan, & Irwin 2007). Two sets of primers were used, first reaction primers (external) BTF1 (forward: 5’ -GGCTCATTACAACAGTTATAG- 3’) and BTR1 (reverse: 5’ -CCCAAAGACTTTGATTTCTCTC- 3’), followed by second reaction primers (internal) BTF2 (forward: 5’ -CCGTGCTAATTGTAGGGCTAATAC- 3’) and BTR2 (reverse: 5’ -GGACTACGACGGTATCTGATCG- 3’). PCRs of both primary and secondary reactions occurred in 25 µL containing 1 µL of DNA template (primary reaction) and 1 µL of the

primary reaction product for the secondary reaction, a working concentration of 0.4 µM of each primer, 1.5 mM of $MgCl_2$, 100 µM of each dNTPs, with 0.02 U/ µL Kapa Taq DNA polymerase (Kapa Biosystems, USA) in reaction buffer (pH 8.5). Cycling conditions were the same for the primary and secondary reactions: an initial denaturation of 5 min at 95 ˚C; 40 cycles with 94 ˚C for 30 s (denaturation), 52 ˚C for 30 s (annealing), and 72˚C for 2 min (extension); and a final extension with 72 ˚C for 7 min. All PCR and sequencing reactions were carried out in an Applied Biosystems (AB) GeneAmp 2720 Thermal Cycler.

10.4.2.2 Trypanosomes

Trypanosomes were detected using a nested PCR method, with trypanosome-specific primers targeting a variable region (~1,500 bp) of the trypanosome small ribosomal subunit RNA gene (18S rDNA), as previously described (McInnes, Hanger, *et al.* 2011). Two fragments (~900 bp each) of the trypanosome 18S rDNA were amplified using two nested PCRs that used one common primer set for the primary (external) amplification, SLF (5' -GCTTGTTTCAAGGACTTAGC- 3') and S762 (5'- GACTTTTGCTTCCTCTAATG- 3'). Two sets of primers for the secondary (internal) amplification were used, S825F (5' -ACCGTTTCGGCTTTTGTTGG- 3') and SLIR (5' -ACATTGTAGTGCGCGTGTC- 3') for the first nested PCR (~959 bp), and S823 (5' -CGAACAACTGCCCTATCAGC- 3') and S662 (5' -GACTACAATGGTCTCTAATC- 3') for the second nested PCR (~904 bp). PCR reactions were carried out in both primary and secondary, in 25 µL reaction volumes containing 1 µL of DNA template (primary reaction) and 1 µL of the primary reaction product for the secondary reaction, a working concentration of 0.8 µM of each primer, 2 mM of $MgCl_2$, 400 µM of each dNTPs, with 0.04 U/ µL Kapa Taq DNA polymerase (Kapa Biosystems, USA) in reaction buffer (pH 8.5). PCR conditions involved a pre-PCR step of 95˚C for 5 min (denaturation), 50 ˚C for 2 min (annealing) and 72 ˚C for 4 min (extension), followed by 35 cycles of 94 ˚C for 30 sec (denaturation), 50 ˚C for 30 sec (primary reaction) 52 ˚C for 30 sec (secondary reaction) (annealing), 72 ˚C for 2 min 20 sec (extension), and a final extension at 72 ˚C for 7 min.

10.4.3 ***Sequencing***

PCR products were visualised in 1% agarose gels made of 0.5x TBE buffer, stained with SYBR-Safe (Life Technologies, Carlsbad, USA) and ran at 90 V, 400 mAmp for ~45 min. Bands of the expected size were cut out using separate sterile scalpel blades and DNA was purified from agarose using the filter tip method. Briefly, 100 µL filter tips were previously prepared by cutting off the bottom so they could fit into a 1.5 mL

microcentrifuge tube (previously UV sterilised) with the lid closed. Excess agarose gel was trimmed off the cut bands and subsequently placed into the filter tips (already inside the Eppendorf tubes). At this stage, microcentrifuge tubes were centrifuged at 16,100 x g for 2 min upon which the filter tip was removed. The filter membrane removed the agarose gel and the resulting flow through contained the purified PCR reaction product.

Sequencing was done at a 1/8 reaction using 1 µL of dye terminator mixture (SABC, Murdoch University) and 1.5 µL of 5x sequencing buffer (SABC, Murdoch University) in a 10 µL final reaction volume given that the concentration of DNA post PCR was within the expected threshold of 5-20 ng as recommended by Applied Biosystems in their BigDye® Terminator v3.1 Cycle Sequencing Kit protocol. Briefly, a master mix containing 1 µL of dye terminator mixture, 1.5 µL of 5x sequencing buffer, 1 µL of 3.2 pmoles working concentration primer (TGV forward primer), and 4.5 µL of PCR grade water (Fisher Biotech, Perth, Australia) was prepared for each PCR product to be sequenced. Lastly, 2-3 µL of purified DNA were added to each reaction, and subsequently vortexed. Cycle conditions were: an initial step of 96 ˚C for 2 m, then 25 cycles of 96 ˚C for 10 s (denaturation), 52 ˚C (annealing) for 5 s, then 60 ˚C for 4 m (extension). PCR products were sequenced in both directions (forward and reverse).

Purification of sequence reaction products was done using the ethanol precipitation protocol for BigDye® Terminator v3.1 (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems, Scoresby, Australia). For each reaction, the entire sequencing reaction product was transferred to a 0.5 mL microcentrifuge tube previously UV sterilised. Subsequently, in the following order, 1 µL of 125mM EDTA, 1 µL of 3M Sodium acetate pH 5.2 and 25 µL of 100% ethanol were added to each reaction tube. Tubes were then briefly vortexed at low speed and left to incubate at room temperature for at least 20 m upon which tubes were centrifuged at maximum speed for 30 min. At this stage, the resulting supernatant was removed using a vacuum pump with P200 filter tips (previously UV sterilised) and tubes were left upside down to dry for at least 3 m. The DNA pellet (not visible) was then gently rinsed with 125 µL of 70% Ethanol, followed by a microfuge step at maximum speed for 5 m at room temperature. Lastly, all supernatant was removed using a vacuum pump with P200 filter tips (previously UV sterilised). Tubes were placed in a Speedvac Concentrator (Savant Instruments, Inc. New York) to dry out and spun for 15 m before being submitted to the sequencing facility at the State Agricultural and Biotechnology Centre (SABC), Western Australia. Sequencing was carried out on an ABI 3790 96 capillary automatic sequencer (Applied Biosystems, Scoresby, Australia).

10.4.4 *Reference intervals for free-ranging females and males on Rottnest Island*

Table 10-2 Haematology reference intervals for males and females of anaesthetised free-ranging quokkas (*S. brachyurus*), sampled on Rottnest Island between March and December 2011. With the exception of platelets, all reference intervals were calculated using the non-parametric method. Negative values of confidence intervals were interpreted as zero.

								Reference Intervals [a]		Confidence Intervals (CI) [b c]			
Parameter	Sex	*n*	Mean [d]	Median [e]	SD [e]	Min [e]	Max [e]	Lower limit	Upper limit	2.5%	5%	90%	97.5%
RBC (x10.e12/L)	f	54	5.68	5.54	0.91	4.14	8.51	4.19	8.29	4.14	4.51	7.45	8.51
	m	59	5.96	5.97	0.82	4.16	7.66	4.35	7.62	4.16	4.64	7.30	7.66
HGB (g/L)	f	54	107	106	17.3	53	149	60.5	148	53	86.4	135	149
	m	59	109	109	13.1	80	140	82.5	138	80	89	130	140
PCV (%)	f	48	33.1	33	4	22	45	23.4	43.9	22	28	38.8	45
	m	53	34	34.1	5.1	20	47	21.4	45.6	20	26.1	41	47
CHCM (g/L)	f	53	305	303	13.8	283	338	283	336	283	286	329	338
	m	59	311	310	15.8	281	342	283	341	281	292	338	342
MCV (fL)	f	54	61.9	61.6	3.45	55.4	70.4	55.5	70.5	55.4	56.9	67.9	70.4
	m	59	59.7	59.6	3.74	52.7	71.2	52.7	69.1	52.7	54	65.5	71.2
Platelet (x10.e9/L)	f [b]	22	508	507	119	322	709	259 †	755 †	197	328	678	817
	m [c]	18	492	478	108	327	813	313 ‡	769 ‡	271	361	651	930
WBC (x10.e9/L)	f	54	4.68	4.26	1.60	2.25	8.70	2.34	8.63	2.25	2.71	7.36	8.70
	m	59	4.60	4.48	1.77	1.75	9.31	1.80	8.70	1.75	2.17	7.70	9.31
Neutrophils (x10.e9/L)	f	52	2.18	2.05	0.74	1.16	3.34	1.16	4.18	1.16	1.25	3.46	4.34
	m	54	2.01	1.95	0.76	0.82	4.65	0.85	4.25	0.82	0.97	3.04	4.65
Lymphocytes (x10.e9/L)	f	53	1.95	1.65	1.12	0.62	5.17	0.63	5.15	0.62	0.72	4.34	5.17
	m	55	2.03	1.92	1.11	0.59	5.36	0.60	4.90	0.59	0.70	3.91	5.36
Monocytes (x10.e9/L)	f	51	0.077	0.058	0.066	**0.00**	0.264	**0.00**	0.262	**0.00**	**0.00**	0.189	0.264
	m	55	0.065	0.056	0.053	**0.00**	0.242	**0.00**	0.219	**0.00**	**0.00**	0.158	0.242
Eosinophils (x10.e9/L)	f	53	0.38	0.30	0.32	0.02	1.38	0.03	1.36	0.02	0.08	1.07	1.38
	m	52	0.29	0.24	0.24	**0.00**	0.96	0.01	0.95	**0.00**	0.04	0.66	0.95
Basophils (x10.e9/L)	f	51	0.02	**0.00**	0.02	**0.00**	0.09	**0.00**	0.09	**0.00**	**0.00**	0.06	0.09
	m	55	0.01	**0.00**	0.02	**0.00**	0.11	**0.00**	0.09	**0.00**	**0.00**	0.05	0.11

Erythrocyte parameters are those of the ADVIA® 120. WBC values are those after correction with nucleated red blood cells. Polymorphonuclear cell values are those of the manual differential count. Min= minimum, Max= maximum, f= female, m= male, RBC= red blood cell concentration, HGB= haemoglobin, PCV= packed cell volume, CHCM= corpuscular haemoglobin concentration mean, MCV= mean corpuscular volume, WBC= white blood cell count

[a] reference intervals computed using the non-parametric method (when n≥ 40), [d] standard method of the untransformed data, [e] robust method of the untransformed data

[b] confidence intervals computed using a bootstrap method (when 20 <n< 120) † reference interval computed using the robust method for the untransformed data

[c] confidence intervals computed using a parametric bootstrap method (when n≤ 20) ‡ reference interval computed using the standard method for the Box-Cox transformed data

Table 10-3 Blood chemistry reference intervals for males and females of anaesthetised free-ranging quokkas (*S. brachyurus*), sampled on Rottnest Island between March and December 2011.

								Reference Intervals [a]		Confidence Intervals [b]			
Parameter	Sex	*n*	Mean [c]	Median [d]	SD [d]	Min [d]	Max [d]	Lower limit	Upper limit	2.5%	5%	90%	97.5%
CK (U/L)	f	52	739	518	690	138	3,109	143	3,080	138	189	1,782	3,109
	m	57	827	366	961	153	4,132	161	3,731	153	194	2,591	4,132
ALT (U/L)	f	53	218	205	56.2	151	374	151.4	362	151	159	321	374
	m	55	204	200	41.6	132	309	134	302	132	150	282	309
ALP (U/L)	f	49	5,988	5,771	3,225	1,387	17,880	1,428	15,995	1,387	1,822	10,120	17,880
	m	54	5,546	5,238	3,014	1,465	17,640	1,551	14,910	1,465	2,072	9,920	17,640
AST (U/L)	f	52	46.5	41.6	21.3	13	126	16.9	118	13	26.7	69	126
	m	56	45.3	39.3	19.3	25	108	25	101	25	26.8	83.9	108
GGT (U/L)	f	40	19.4	19.4	6.4	8	30	8	30	8	11	29	30
	m	42	15.7	15.2	4.4	8	29	8.1	28.6	8	10.1	22.8	29
Protein (g/L)	f	53	60.6	60.4	4.68	50.5	72.3	51.4	71.5	50.5	53.8	67.8	72.3
	m	58	59.9	59.7	4.71	49.5	71.5	50.1	70.6	49.5	52.4	67.7	71.5
Albumin (g/L)	f	53	36.2	36.3	1.90	31.7	41.1	31.8	40.5	31.7	32.7	38.9	41.1
	m	58	36.2	36.5	1.99	30	40.6	31	40.2	30	32.9	38.8	40.6
Globulin (g/L)	f	53	24.4	24.2	3.90	14.4	36	15.5	34.2	14.4	18.9	29.9	36
	m	58	23.7	23.5	3.76	15.1	32.8	15.5	32.6	15.1	17.9	30	32.8
Glucose (mmol/L)	f	53	4.85	4.64	2.41	0.7	12.5	0.77	11.9	0.70	1.31	8	12.5
	m	58	3.51	3.11	1.84	0.6	9.7	0.60	8.9	0.60	1.48	6.10	9.7
Cholesterol (mmol/L)	f	53	2.91	2.90	0.53	2	4	2.04	3.90	2	2.14	3.70	4
	m	58	2.75	2.75	0.56	1.5	3.9	1.64	3.85	1.50	1.95	3.50	3.90
Bilirubin (µmol/L)	f	53	4.19	3.95	1.63	1	9	1.35	8.55	1	2	7.13	9
	m	57	4.36	4.02	1.86	1.2	9.4	1.56	9.22	1.20	2	8.10	9.40
Urea (mmol/L)	f	53	7	6.83	1.34	4.8	10.3	4.91	10.2	4.80	5.37	9.20	10.3
	m	57	6.80	6.64	1.61	4.2	12.3	4.20	11.5	4.20	4.39	9	12.3
Creatinine (µmol/L)	f	52	73.8	71.3	17.1	41	112	43.3	112	41	51.7	105	112
	m	58	69	66.7	13.9	47	107	47	104	47	49.4	94.6	107
Phosphorus (mmol/L)	f	53	1.07	1.01	0.48	0.4	2.8	0.40	2.49	0.40	0.54	1.80	2.80
	m	58	1.32	1.28	0.43	0.5	2.5	0.55	2.41	0.50	0.70	2.01	2.50
Calcium (mmol/L)	f	53	2.22	2.22	0.22	1.63	2.69	1.69	2.67	1.63	1.93	2.56	2.69
	m	58	2.18	2.17	0.18	1.76	2.67	1.69	2.58	1.76	1.89	2.46	2.67
Vitamin E (mg/L)	f	52	6.94	5.90	1.77	4.05	11	4.12	10.6	4.05	4.36	9.62	11
	m	56	6.19	5.90	1.92	3.84	10.9	3.90	10.6	3.84	4.13	9.48	10.9

CK= creatinine kinase, ALT= alanine aminotransferase, ALP= alkaline phosphatase, AST= aspartate aminotransferase, GGT= gamma-glutamyl transferase; [a] Reference Intervals computed using the non-parametric method (when n≥ 40), [b] Confidence Intervals computed using a bootstrap method (when 20 <n<120), [c] standard method of the untransformed data, [d] robust method of the untransformed data.