Urbanisation and small marsupials in the greater Perth region, Western Australia

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Wee, sleekit, cowrin, tim'rous beastie,
O, what a panic's in thy breastie!
Thou need na start awa sae hasty,
              Wi' bickering brattle!
I wad be laith to rin an' chase thee,
              Wi' murd'ring pattle!

I'm truly sorry Man's dominion
Has broken Nature's social union,
An' justifies that ill opinion,
              Which makes thee startle,
At me, thy poor, earth-born companion,
              An' fellow-mortal!

I doubt na, whyles, but thou may thieve;
What then? poor beastie, thou maun live!
A daimen icker in a thrave
              'S a sma' request.
I'll get a blessin wi' the lave,
              An' never miss't!

Thy wee-bit housie, too, in ruin!
Its silly wa's the win's are strewin!
An' naething, now, to big a new ane,
              O' foggage green!
An' bleak December's winds ensuin,
              Baith snell an' keen!

Thou saw the fields laid bare an' waste,
An' weary Winter comin fast,
An' cozie here, beneath the blast,
              Thou thought to dwell,
Till crash! The cruel coulter past
              Out thro' thy cell.

That wee-bit heap o' leaves an' stibble,
Has cost thee monie a weary nibble!
Now thou's turn'd out, for a' thy trouble,
              But house or hald,
To thole the Winter's sleety dribble,
              An' cranreuch cauld!

But, Mousie, thou art no thy lane,
In proving foresight may be vain:
The best-laid schemes o' Mice an' Men
              Gang aft a-gley,
An' lea'e us nought but grief an' pain,
              For promis'd joy!

Still thou are blest, compar'd wi' me!
The present only toucheth thee:
But, Och! I backward cast my e'e
              On prospects drear!
An' forward, tho' I canna see,
              I guess an' fear!

-Robert Burns

Author's Declaration

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

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Alison Elizabeth Hillman

Abstract

Background

Quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*) remain in many areas of Perth, Western Australia, despite urbanisation. This thesis investigated the impacts of urbanisation on the parasitic infections, morphometrics and reproductive fitness of these marsupials. Parasite screening particularly investigated the presence of parasites that quenda and possums may be acquiring from humans and pets in urbanised environments.

Methods

This cross sectional study targeted free-ranging quenda and brushtail possums in Perth. Trapping was undertaken on 29 bushland sites and 35 urbanised sites. Trapped marsupials were sampled for gastrointestinal and macroscopic ecto- parasites and *Toxoplasma gondii* antibodies, and morphometrics were measured. Statistical analyses included linear and logistic regression, Kulldorff's spatial scan statistic and analysis of similarities in parasite communities.

Results

287 quenda and 33 brushtail possums were sampled across 55 sites. Parasitic infections were common in Perth quenda, and less so in brushtail possums. Twelve parasite taxa were identified infecting these host species for the first time. In quenda, some differences in parasite prevalences, odds of infection, intensities of infection, and parasite communities, were observed in quenda trapped in urbanised environments compared to bushland. In quenda, *Giardia* spp. infection was common, and typically the non-zoonotic *G. peramelis*. Analyses suggest that the primary influence on the distribution of *Giardia* spp. infection in quenda is population density. Quenda trapped in urbanised environments were heavier on average than quenda trapped in bushland, in the absence of convincing evidence of a difference in skeletal morphometrics.

Bushland adult female quenda had increased odds of having an active pouch than those in urbanised environments.

Conclusions

Quenda and brushtail possums in Perth are not commonly infected with gastrointestinal and macroscopic ecto- parasites of humans and domestic animal origin. Quenda in urbanised environments have a tendency towards obesity. Urbanised environments may negatively impact fecundity of female quenda.

Acknowledgements

Undertaking an epidemiological study of wildlife has been both an immense privilege and a heavy burden of responsibility, in terms of interfering with free-ranging wildlife to collect data. This research is dedicated to my family - particularly my parents Mary and David - and to my colleagues at the Parasitology Group. Without their boundless support it would not have been achieved.

Finally, to the beasties themselves - it was an honour! I hope I did you proud.

List of publications, and works in progress, included in this thesis

Chapter 1.4

Hillman, A.E., Lymbery, A.J., Thompson R.C.A., 2016. Is *Toxoplasma gondii* a threat to the conservation of free-ranging Australian marsupial populations?. *International Journal for Parasitology: Parasites and Wildlife*, 5, 17-27.

Chapter 3

Hillman, A., Thompson, R.C.A., 2016. Interactions between humans and urban-adapted marsupials on private properties in the greater Perth region. *Australian Mammalogy*, 38, 253-255.

Chapter 4

Hillman, A.E., Ash, A.L, Kristancic, A.R., Elliot, A.D., Lymbery, A.J., Robertson, I.D., Thompson, R.C.A. Validation of various parasite detection tests for use in the Australian marsupials quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*). *Journal of Veterinary Diagnostic Investigation*, In press*.*

Chapter 5

Hillman, A.E, Lymbery, A.J, Robertson, I.D, Thompson, R.C.A. Biosecurity and cross-contamination in epidemiological studies involving trapping and sampling wildlife. *Short communication, submitted to journal.*

Chapter 6

Hillman, A.E, Lymbery, A.J, Elliot, A.D, Ash, A.L, Thompson, R.C.A. Parasitic infections of Perth brushtail possums (*Trichosurus vulpecula*). *For submission to journal.*

Chapter 7

Hillman, A.E, Lymbery, A.J, Elliot, A.D, Thompson, R.C.A. Urbanisation and the quenda: parasites, proportions and parity. *For submission to journal.*

Chapter 8

Hillman, A.E, Lymbery, A.J, Thompson, R.C.A. Anthropozoonotic significance, risk factors and spatial distribution of *Giardia* spp. infection in quenda (*Isoodon obesulus*) in the greater Perth region, Australia. *For submission to journal.*

Chapter 9

Hillman, A., Ash, A., Elliot, A., Lymbery, A., Perez, C., Thompson, R.C.A., 2016. Confirmation of a unique species of *Giardia*, parasitic in the quenda (*Isoodon obesulus*). *International Journal for Parasitology: Parasites and Wildlife*, 5, 110-115.

Chapter 10

Hillman, A.E, Yang, R., Lymbery, A.J., Thompson, R.C.A. *Eimeria* spp. infections quenda (*Isoodon obesulus*) in the greater Perth region, Western Australia. *Submitted to journal.*

Statement of human and animal ethics approval

The protocols for data collection from Perth quenda and brushtail possums, and from Perth pet cats, were reviewed and approved by the Murdoch University Animal Ethics Committee (permit number R2530/12).

The protocols for surveying cat owners, and private property owners with backyard marsupials, were reviewed and approved by the Murdoch University Human Research Ethics Committee (project numbers 2012/180 and 2013/167, respectively).

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List of Abbreviations

18s rRNA	18S ribosomal RNA locus
bg	β-giardin locus
CI	Confidence interval
CrI	Credible interval
ELISA	Enzyme linked immunosorbent assay
FFP	Faecal flotation protocol
gdh	Glutamate dehydrogenase locus
GI	Gastrointestinal
IMF	Immunofluorescence microscopy
MAT	Modified agglutination test
OD	Optical density
OR	Odds ratio
PCR	Polymerase chain reaction
SG	Specific gravity
tpi	Triose phosphate isomerase locus
vs.	Versus

Introduction to thesis

This thesis comprises a literature review and nine inter-related papers. Four published papers (Chapter subsection 1.4, and Chapters 3, 4 and 9) have been presented in this thesis with minor formatting changes. A further five papers have been submitted to journals, or are intended for submission. They are presented as Chapters 5, 6, 7, 8 and 10, and as such there is some duplication of material in the introductions and methodology.

Chapter 1 reviews the literature on the impacts of urbanisation on marsupial wildlife, with a focus on the host species quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*), and the parasitic infections *Giardia* spp. and *Toxoplasma gondii*. The aims and objectives of this research are then presented.

Chapter 2 reports the findings of a cross-sectional study of Perth pet cats and cat owners, undertaken to clarify the potential role of Perth pet cats in the epidemiology of *Toxoplasma gondii* in the greater Perth region.

Chapter 3 reports the findings of a survey of Perth private property owners and residents, undertaken to identify the types of interactions that occur between humans and quenda and brushtail possums on private properties in the greater Perth region.

Chapter 4 presents the results of a study aiming to validate the use of various parasite detection tests in quenda and brushtail possums.

Chapter 5 outlines the approaches taken to biosecurity in the quenda and brushtail possum cross sectional studies (as per Chapters 6, 7, 8, 9 and 10), and the evidence to suggest that the biosecurity procedures were effective at minimising the impact of cross-contamination of samples on the cross sectional study findings.

Chapter 6 presents the findings of a cross sectional study of free-ranging brushtail possums in the greater Perth region. This includes comparisons of parasite prevalences, subjective body condition and pouch activity of brushtail possums trapped in urbanised environments compared to those trapped in bushland.

Chapter 7 presents the findings of the cross sectional study of free-ranging quenda in the greater Perth region. This study compared: parasite prevalences and intensities of infection, and parasite communities, between quenda trapped in urbanised environments and bushland; morphometrics of adult quenda trapped in urbanised environments and bushland; and pouch activity of adult female quenda trapped in urbanised environments and bushland.

Chapter 8 expands on the results presented in Chapter 7, by investigating the epidemiology of *Giardia* spp. infection in quenda in the greater Perth region.

Chapter 9 expands on findings of Chapter 8, in formally describing the 'quenda genotype' of *Giardia* as a unique species, *Giardia peramelis*.

Chapter 10 expands on the findings of Chapter 7, by investigating the species of coccidia (Family Eimeriidae) present in Perth quenda. This includes formal description of a novel species of *Eimeria* as *Eimeria angustus*, and molecular characterisation of *Eimeria angustus* and *Eimeria kanyana.*

Chapter 11 presents overall conclusions regarding the potential impacts of urbanisation on quenda and brushtail possums in the greater Perth region. This includes observations and hypotheses generated by these research findings, in consideration of other research findings regarding the impacts of urbanisation on mammalian species worldwide. Limitations of this research are discussed, with reference to potential approaches to future research to clarify and expand on the findings of this study.

Appendices A and B present images of parasites identified from quenda and brushtail possum hosts, respectively, as part of this research. They are intended to illustrate these research findings.

Chapter 1

General Introduction

A portion of this chapter is derived from a published review, with some formatting modifications:

Hillman, A.E., Lymbery, A.J., Thompson, R.C.A., 2016. Is *Toxoplasma gondii* a threat to the conservation of free-ranging Australian marsupial populations? *International Journal for Parasitology: Parasites and Wildlife*, 5, 17-27.

The remaining material is the sole work of the thesis author.

1. General introduction

1.1 Urbanisation and wildlife

Increasing urbanisation - the proportion of people residing in cities and the land coverage of cities - is a worldwide phenomenon. Globally, 54% of the world's population live in cities, and this is estimated to rise to 66% by 2050 (United Nations Department of Economic and Social Affairs, 2014). In Australia, population growth continues (Australian Bureau of Statistics, 2016), and with it urbanisation. Most recent estimates indicate that over two thirds of the Australian population reside in major cities (Australian Bureau of Statistics, 2006).

1.1.1 The impacts of urbanisation on wildlife

Urbanisation has important ecological effects. It can cause local extinction of wildlife populations as a result of habitat loss, with a resulting decline in species richness (McKinney, 2002; Garden et al., 2006). Some wildlife species are capable of surviving in urbanised environments, and urbanisation may impact their population health in a variety of other ways.

For example, wildlife in urbanised environments are at risk of acquiring infections from humans and domestic pets (Thompson, 2013; Soulsbury and White, 2016). This is of concern from two perspectives: 1) the impact of such infections on wildlife population health; and 2) the potential for wildlife to then become reservoirs of infections that are capable of infecting humans and domestic animals (Daszak et al., 2000; Bradley and Altizer, 2007). The impacts on wildlife population health are directly relevant to urban dwelling wildlife populations, and also of relevance regarding urban to non-urban translocation of wildlife. In this circumstance, translocated animals have the potential to act as vectors in the spread of human and domestic animal-based infections to wildlife

populations which may not otherwise be exposed to such pathogens (Cunningham, 1996).

Additionally, wildlife in urban areas may have access to anthropogenic food sources. An abundance of food sources in urbanised environments may lead to abnormally increased population densities, which may result in increased infection transmission within wildlife populations and increased aggression (Daszak et al., 2000, Bradley and Altizer, 2007, Brearley et al., 2013). Anthropogenic food sources may result in juvenile animals becoming dependent on such food, and may also result in nutritional imbalances that can lead to metabolic disorders and obesity - though there is not yet sufficient empirical evidence to support this (Orams, 2002).

1.1.2 The impacts of urbanisation on the epidemiology of parasitic infections in Australian marsupial species

The impact of urbanisation on urban dwelling marsupial species in Australia is an area of limited investigation. From the perspective of parasitic infections, several studies have compared ectoparasitism, *Cryptosporidium* spp. infection and *Toxoplasma gondii* seroprevalence between brushtail possums (*Trichosurus vulpecula*) in urbanised environments in Sydney, and geographically remote bushland in New South Wales (Hill et al., 2008a, b; Webster et al., 2014), and ectoparasitism between long-nosed bandicoots (*Perameles nasuta*) in suburban backyards and bushland within the greater Sydney region (Dowle, 2012). Further data have been obtained on *T. gondii* seroprevalence in brushtail possums in urbanised environments in Sydney and geographically remote bushland in New South Wales, *Cryptosporidium* spp. infections in southern brown bandicoots (*Isoodon obesulus*) and long-nosed bandicoots in urbanised environments in Sydney, and *Giardia* spp. infections in quenda (syn. southern brown bandicoots, *I. obesulus*) in urbanised Perth and bushland in Western Australia (Eymann, 2006; Thompson et al., 2010a; Dowle et al., 2013). However, the

results of all previous investigations are prone to substantial limitations, including: small sample sizes; selection bias, particularly as a result of sampling few bushland and / or urbanised sites in undertaking comparisons; misclassification bias, particularly in classifying animals as infected or not infected with various parasites using unvalidated methodology; Type I error, due to potential data correlation which was not considered in statistical analyses; and a lack of consideration of the potential impacts of confounding on results.

A study comparing ectoparasitism in brushtail possums sampled in urbanised environments in Sydney to those sampled in a New South Wales bushland reserve found no difference in ectoparasite species richness between brushtail possums in urbanised environments compared to bushland. The flea *Echidnophaga myrmecobii* and tick *Ixodes trichosuri* were more prevalent in brushtail possums trapped in urbanised environments, and conversely the mite *Atellana papilo* was more common in brushtail possums trapped in bushland (Webster et al., 2014). However, in addition to limitations as described above, 35.6% of the data were repeat samples of individual possums, and despite this all data were treated as independent observations in statistical analyses. This entails an increased risk of Type I error in these results. Further, sampling from relatively limited geographic areas in both the urbanised environments and bushland leaves these findings prone to selection bias, and environmental conditions that differ between the urbanised sites and bushland site may have been a further source of selection bias, as the urbanised sites and bushland site were geographically remote from each other.

In a study of *Cryptosporidium* spp. infections in brushtail possums from urbanised Sydney and a fenced woodland population in New South Wales, 11.3% (Jeffrey's 95% CI 7.0 – 17.8%) of urban animals were positive for *Cryptosporidium* spp., compared to 5.6% (Jeffrey's 95% CI 1.3 – 26.0%) of woodland animals (Hill et al., 2008a). All sequenced isolates were of novel parasite genotypes that are not known to infect

humans or domestic animals. Descriptively, these findings suggest that high population densities in urbanised Sydney may be the cause of the relatively higher prevalence of *Cryptosporidium* spp. infections. However, as per the limitations listed above, the relatively small sample sizes mean that the discrepancy in prevalence estimates cannot be confidently asserted to be more than a chance finding (χ^2 test p = 0.46), and limited geographical distribution of sampling sites in both urbanised environments and in bushland mean that these findings may not be representative of the impact of urbanisation on the epidemiology of *Cryptosporidium* spp. infections in brushtail possums in Sydney. Additionally, as above, the comparison of brushtail possums in urbanised environments to bushland may have been biased by the geographic remoteness of the urbanised sites and bushland site, if environmental variables of relevance to the epidemiology of *Cryptosporidium* spp. infections in brushtail possums differ between those sites. Further, the bushland brushtail possums to which the urbanised brushtail possums were compared, were from a population within predator proof fencing. This may have influenced population dynamics, and thus altered infection epidemiology at the bushland site, which may have been an additional source of bias.

Using the modified agglutination test at a titre cut-off of ≥ 1:25, both Eymann et al. (2006) and Hill et al. (2008b) identified antibodies to *T. gondii* in brushtail possums trapped in urbanised environments in Sydney (seroprevalences were 6.7%, Jeffrey's 95% CI 3.6 – 12.2%; and 2.4%, Jeffrey's 95% CI 2.2 – 10.0%, respectively). Conversely, of brushtail possums trapped in bushland in these studies, none had *T. gondii* antibodies in either study (0%; Jeffrey's 95% CI 0 – 36.9% and 0 - 18.5%, respectively). However, the first study (Eymann et al., 2006) was not designed to compare *T. gondii* infection prevalence in urbanised environments compared to bushland, and so the use of these data to do so entails many limitations, which also apply to the second study (Hill et al., 2008b). These include all limitations described above, and in particular the comparison of brushtail possums in urbanised

environments to geographically remote bushland, leaving the findings prone to selection bias.

Dowle (2012) compared ectoparasitism between long-nosed bandicoots trapped in suburban backyards and two bushland sites in the greater Sydney region, and found that tick prevalence (Order Ixodida) was lower in one bushland site, compared to the similarly higher prevalences of animals trapped at the second bushland site and in suburban backyards. However, it appears that repeat measures data were used and not accounted for statistically. If so, this would have entailed a relatively increased risk of Type I error in these findings, additional to the previously-described limitations. There was no evidence of a difference in prevalence of other ectoparasites between the bandicoots trapped in suburban backyards and the two bushland sites.

Dowle et al. (2013) investigated *Cryptosporidium* spp. infections in southern brown bandicoots and long-nosed bandicoots inhabiting urbanised environments in Sydney. The *Cryptosporidium* spp. isolates were not able to be fully characterised; however, the 18S rRNA locus isolates sequenced were most closely related to *C. parvum* and *C. hominis*. This is suggestive, though not conclusive, that the source of *Cryptosporidium* spp. infection in these bandicoots may have been anthropogenic.

Thompson et al. (2010a) sampled quenda from urban Perth as part of a survey of Western Australian wildlife for *Giardia* spp. Nine out of 14 quenda (64.3%; Jeffrey's 95% CI 38.4 – 83.7%) from urbanised environments were positive for *Giardia* spp., compared to 5 / 41 (12.2%; Jeffrey's 95% CI 5.4 – 25.6%) quenda from three bushland sites. *Giardia duodenalis, G. canis* and the 'quenda genotype' were sequenced from urban quenda, and *Giardia bovis* and the 'quenda genotype' were sequenced from quenda in bushland. This suggests that domestic pets and humans in urban environments may be sources of *Giardia* spp. infection to quenda in urbanised Perth, but again limitations of sample size and potential influences of sampling bias require

more extensive studies to quantify the extent of this transmission of infection, and the impact on quenda population health.

1.1.3 Other impacts of urbanisation on Australian marsupial species

One study has made observations on the impact of urbanisation on reproduction in an Australia marsupial species. Statham and Statham (1997) documented births throughout the year in a population of brushtail possums in urbanised Launceston, Tasmania. This contrasts to seasonal breeding of brushtail possums in non-urbanised environments in Tasmania, and was attributed to abundant nutritional resources available in urbanised environments maintaining fertility throughout the year (Statham and Statham, 1997).

Another study noted higher mean body condition in long-nosed bandicoots trapped in the suburban backyards in northern Sydney, compared to two bushland sites in the greater Sydney region (Dowle, 2012). However, the potential data correlation was not considered in these analyses, and the lack of validation in the body condition measurement is another substantial limitation to this finding.

1.2 Small marsupials in urban Perth, Western Australia

In Perth, Western Australia, the marsupials quenda and brushtail possums remain in many areas despite urbanisation (Howard et al., 2014; Department of Parks and Wildlife, 2015).

1.2.1 Quenda (syn. southern brown bandicoots, *Isoodon obesulus*)

Quenda (Figure 1.1) is a common name used to refer to *I. obesulus* in Western Australia*;* "southern brown bandicoot" tends to be used as the common name used for *I. obesulus* elsewhere in Australia. Molecular taxonomic studies of the genus *Isoodon* suggests that *I. obesulus* in Western Australia form a clade with most South Australian mainland and island *I. obesulus* populations, and with golden bandicoots (*Isoodon auratus*) (conspecific status of these two species is justified, based on genetic data). *Isoodon obesulus* populations in New South Wales, Victoria, Tasmania and south east South Australia comprise a second, distinct clade (Pope et al., 2001; Zenger et al., 2005; Cooper et al., 2015).

Figure 1.1 Quenda (*Isoodon obesulus*)

Quenda / southern brown bandicoots are small, omnivorous, terrestrial marsupials. In bushland, they primarily eat invertebrates, as well as tubers and fungi (Quin, 1988), and each individual will turn over up to approximately four tonnes of soil per year in foraging for these foodstuffs (Valentine et al., 2013). They will also scavenge meat (Heinsohn,1966; O'Brien et al., 2010), and are known to be capable of killing a variety of small prey species in experimental conditions (Moloney, 1982). In urban areas of Perth, Australia, they are known to scavenge, or be offered by members of the public, a variety of other foodstuffs (Howard et al., 2014).

In bushland, quenda / southern brown bandicoots typically construct nests on the ground amongst dense vegetation, and may take refuge amongst dense vegetation or in the burrows of other animals (Broughton and Dickman, 1991). They also appear to be capable of digging burrows, though it is uncertain whether this is common or occurs only in exceptional circumstances, such as sheltering after fires (Long, 2009).

Anthropogenic variations in nest and refuge locations in urbanised environments may occur, such as nesting under buildings, but have not been formally documented. They may have more than one nest (Lobert, 1990) and multiple individuals have been observed occupying the same refuges (Broughton and Dickman, 1991). While they have a tendency to crepuscular / nocturnal activity, they are known to also be active during the day (Heinsohn, 1966; Lobert, 1990; Larcombe, 2003; Howard et al., 2014).

In bushland within the Statistical Division of Perth, the home range of quenda may vary from 0.28 - 8 ha. Average home ranges vary with season - up to 2.19 ha (females) and 2.85 ha (males) (Broughton and Dickman, 1991). There are conflicting data as to whether or not quenda / southern brown bandicoots are solitary and territorial; territoriality and home range may be inversely related to factors such as the availability of resources in the area (Heinsohn, 1966; Lobert, 1990; Broughton and Dickman, 1991).

Quenda / southern brown bandicoots are weaned at approximately 60 days, and rapidly develop to sexual maturity by four to seven months of age (Heinsohn, 1966; Lobert and Lee, 1990). Year-round breeding has been demonstrated in South Australian populations (Copley et al., 1990; Sanderson and Kraehenbuehl, 2006), however breeding appears to be seasonal in populations in Tasmania, Victoria and New South Wales (Heinsohn, 1966; Stoddart and Braithwaite, 1979; Lobert and Lee, 1990; Dowle, 2012). Females have an oestrous period during late lactation, allowing new pouch young to replace the previous litter once weaned (Heinsohn, 1966). In light of this high reproductive potential, there has been some speculation as to whether the species may have an intrinsic population regulation mechanism induced by stress associated with high population density (Thomas, 1990). However, this hypothesis has not been specifically investigated.

The maximum lifespan of free-ranging quenda / southern brown bandicoots is thought to be 3.5 years (Lobert and Lee, 1990). In captivity, they may live longer - a lifespan of six years has been documented, though survival past five years is rare (L. Kelman, Perth Zoo - pers. comm.).

A variety of interactions between quenda and humans are known to occur in urban areas (K. Howard, WWF-Australia, pers. comm.). Surveys suggest that the majority of members of the public in Brisbane, Sydney and Perth (Australia) have positive attitudes towards *Isoodon* spp. and other bandicoots (Family Peramelidae) in urban areas. However, negative attitudes are not uncommon, and non-response bias cannot be excluded due to low survey response rates. Negative attitudes tended to be particularly associated with annoyance at the holes dug in gardens by bandicoots foraging for food, and concerns about ticks associated with the animals (FitzGibbon and Jones, 2006; Dowle and Deane, 2009; Howard et al., 2014). Surveys conducted by FitzGibbon and Jones (2006) and Howard et al. (2014) confirmed that some members of the public undertake unauthorised translocations of quenda / southern brown bandicoots from their property, despite this being illegal (e.g. in Western Australia - Wildlife Conservation Act, 1950).

1.2.2 Brushtail possums (*Trichosurus vulpecula*)

Brushtail possums (Figure 1.2) are medium sized arboreal marsupials. The subspecies found in south west Western Australia (which includes the greater Perth region) is *T. vulpecula hypoleucus*, with five other *T. vulpecula* subspecies found Australia-wide (Van Dyck and Strahan, 2008).

Figure 1.2 Brushtail possum (*Trichosurus vulpecula*)

Brushtail possums are omnivorous. In non-urbanised environments they have a primarily herbivorous diet (Evans, 1992; How and Hillcox, 2000), though they are known to opportunistically scavenge meat (O'Brien et al., 2010), but in urbanised environments they will scavenge a wide variety of foods, including scraps, fruit from garden trees and introduced garden plant species (McDonald-Madden et al., 2000; Kerle, 2001).

Brushtail possums are strictly nocturnal. In non-urbanised environments, they typically nest in tree hollows (How and Hillcox, 2000) and usually have more than one den (Statham and Statham, 1997; Harper, 2005). In the absence of trees with hollows, they may nest in cracks and crevices (Kerle, 2001). In urbanised environments, brushtail possums often nests in dark recesses associated with buildings (in or under buildings, in roof cavities, or in chimneys (Statham and Statham, 1997).

Brushtail possums are generally solitary. In tuart forest in south west Western Australia, females have been estimated to have an average home range of 1.26 ha, with males having a larger average home range of 2.21 ha (How and Hillcox, 2000). Home ranges have been estimated to be much greater in south-west Western Australian jarrah forest (Cruz et al., 2012), but home ranges in more urbanised settings in Western Australia have not been documented. In an urbanised environment in Tasmania, the average home ranges have been estimated at 2.0 ha (females) and 10.9 ha (males), respectively (Statham and Statham, 1997). In contrast, in peri-urban bushland in Victoria average home ranges have been estimated at 1.02 ha (females) and 1.19 ha (males) (Harper, 2005). The marked variation in these findings suggests that site-specific factors, particularly local environmental conditions, are important influences on home range (Bennett et al., 1991; Harper, 2005).

Brushtail possums are weaned at six to seven months of age, and reach sexual maturity by 12 - 14 months. In temperate regions, such as the greater Perth region, they breed seasonally, with a peak of births in autumn, and a lesser peak of births in spring (Kerle, 2001). The typical lifespan of brushtail possums is up to 11 years, but they may live up to 13 years (Van Dyck and Strahan, 2008).

Community attitudes to urban dwelling possums vary, with people expressing both positive and negative sentiments. Three surveys of members of the public in Sydney found negative attitudes are mostly founded on possums inhabiting roof spaces and

subsequently causing property damage and noise (Hill et al., 2007; Russell et al., 2011; Wilks et al., 2013). Though the majority of respondents had generally positive attitudes towards possums, non-response bias cannot be excluded due to relatively low response rates. These surveys indicated that brushtail possums are sometimes - and possibly commonly - illegally translocated from private properties, as for quenda. Whilst the release location was often not described, bushland locations were nominated in some cases (Hill et al., 2007; Russell et al., 2011; Wilks et al., 2013).

1.3   *Giardia* spp. infections in Australian marsupial species

Giardia is a genus of flagellate protozoan parasites. Anthropozoonotic spread of *Giardia* spp. may be of concern regarding free-ranging marsupial species living in urbanised environments in Australia (Thompson et al., 2010a; Thompson, 2013). However, as yet there have been no extensive studies of urban dwelling marsupial populations to quantify the extent and possible impact of this genus of parasites on these hosts (Appelbee et al., 2005; Thompson et al., 2010b).

1.3.1   *Giardia* spp. taxonomy

Giardia species-level taxonomy has changed markedly over a relatively short time frame. This thesis has adopted the most recently proposed revision of *Giardia* taxonomy (Monis et al., 2009) (Table 1.1). All articles referred to in this thesis have been taxonomically translated, corresponding to the revised *Giardia* taxonomy system.

Table 1.1 *Giardia* spp. taxonomy: the immediately superseded and revised taxonomy systems for the *Giardia* genus

Superseded *Giardia* taxonomy: *G. duodenalis* as a 'species complex':		Revised *Giardia* taxonomy: *G. duodenalis* assemblages as separate species:	Primary host species
G. duodenalis	Assemblage A	*Giardia duodenalis*	
	-Subassemblage AI	-Genotype AI	Humans, other mammals
	-Subassemblage AII	- Genotype AII	Humans
	-Subassemblage AIII	- Genotype AIII	Wild ungulates
	Assemblage B	*Giardia enterica*	
	-Subassemblage BIII	- Genotype BIII	Humans, other mammals
	-Subassemblage BIV	- Genotype BIV	Humans
		Giardia canis	
	Assemblage C	- Genotype C	Dogs
	Assemblage D	- Genotype D	Dogs
	Assemblage E	*Giardia bovis*	Cattle, other ungulates
	Assemblage F	*Giardia cati*	Cats
	Assemblage G	*Giardia simondi*	Rats and mice
	Assemblage H[1]	'Pinniped genotype'[1]	Pinnipeds (seals)
'Quenda genotype'[1]		'Quenda genotype'[1]	Quenda
Giardia microti		*Giardia microti*	Rodents
Giardia muris		*Giardia muris*	Rodents
Giardia psittaci		*Giardia psittaci*	Birds
Giardia ardeae		*Giardia ardeae*	Birds
Giardia agilis		*Giardia agilis*	Amphibians

[1] Novel lineages - not formally described, though likely novel species

1.3.2   *Giardia* spp. lifecycle

Species of *Giardia* infect the intestinal tract, and have a direct lifecycle (Feng and Xiao, 2011). After ingestion by the host, *Giardia* spp. cysts excyst in the duodenum, releasing trophozoites. Alternatively, but rarely, the host may be infected by directly ingesting trophozoites (Thompson and Monis, 2012). Trophozoites multiply by binary fission in the anterior small intestine. There is evidence to suggest that recombination occurs as part of the lifecycle. However, the frequency and its influence on genetic diversity in the genus remains unknown (Caccio et al., 2008; Sprong et al., 2009; Thompson and Monis, 2012). When passed to the ileum, conditions stimulate the trophozoites to secrete a resistant wall and encyst. Cysts then pass out in the faeces, and are immediately infectious to other hosts (Feng and Xiao, 2011). Infected hosts shed cysts

in their faeces intermittently (Rosa et al., 2007; Thompson (R) et al., 2008). *Giardia* spp. cysts remain viable in the environment for months in cool, damp conditions. Cysts are resistant to chlorine, but sensitive to desiccation, high temperatures, freezing, sunlight, and some disinfectants (Acha and Szyfres, 2003; Feng and Xiao, 2011; Leder and Weller, 2012). Particularly where diarrhoea is present, trophozoites may also be excreted in the infected host's faeces, pre encystation (Acha and Szyfres, 2003). Trophozoites are capable of infecting in a new host, but cannot survive for long in the external environment (Rosa et al., 2007; Thompson and Monis, 2012).

1.3.2.1 *Giardia* spp. transmission and infection

Giardia spp. cysts are transmitted via the faecal-oral route. Contaminated water is a major source of transmission for humans, and cysts can also be spread via contaminated food and fomites (Acha and Szyfres, 2003; Feng and Xiao, 2011; Leder and Weller, 2012).

The infectious doses of *G. duodenalis* and *G. enterica* are low, at approximately 10 to 25 cysts for humans (Leder and Weller, 2012). Prepatent periods for *Giardia* spp. can vary from 3 to 21 days (Thompson and Monis, 2012). Some infections self-resolve; others, if left untreated, may persist for up to two years (Wolfe, 1992). A host may be infected with multiple species or genotypes of *Giardia* concurrently (e.g. Upcroft and Upcroft, 1994; Geurden et al., 2008). Approximately 50% of exposed humans shed *Giardia* spp. cysts in their faeces, and the number shed daily can be very high (up to 900 million cysts per day) (Acha and Szyfres, 2003). Animals are susceptible to reinfection throughout life, though previously infected individuals may develop some degree of immunity (Wolfe, 2008).

Most species and genotypes of *Giardia* are host-adapted (Table 1.1), though occasional 'spill over' of host-adapted types to different host species has been documented (Feng and Xiao, 2011; Table 1.2). Humans are believed to be the principal

reservoir responsible for human giardiasis (Acha and Szyfres, 2003). In Australia, survey results suggest that canine and feline *Giardia* spp. infections are dominated by the host-adapted *G. canis* and *G. cati,* respectively (Hopkins et al., 1997; Palmer et al., 2008).

However, *G. duodenalis* genotype AI and *G. enterica* genotype BIII are known to infect a wide range of mammals, including humans, cats and dogs (Thompson, 2004; Feng and Xiao, 2011) (Tables 1.1 and 1.2). As a result, infections in a variety of host species may be relevant to the transmission of these *Giardia* spp. infections in a given location. Zoonotic *G. duodenalis* infections identified in a variety of wildlife species previously have been linked epidemiologically to a human source of infection, either directly or via domestic animal hosts (Thompson et al., 2009; Thompson et al., 2010b). However, a lack of data precludes quantification of the magnitude of risk humans, domestic animals and wildlife pose to each other regarding transmission of the anthropozoonotic *Giardia* spp. infections (Caccio and Ryan, 2008; Thompson et al., 2010b; Feng and Xiao, 2011).

In Western Australia, limited investigations have identified *G. duodenalis* in quenda faeces (Thompson et al., 2010a). However, insufficient data exist to elucidate their role, if any, in the epidemiology of anthropozoonotic *Giardia* spp. infections in urbanised environments. *Giardia* spp. has not been identified infecting free-ranging brushtail possums in Western Australia in previous investigations (Adams, 2003; Thompson et al., 2010a).

Table 1.2 *Giardia* spp. known to infect humans, dogs, cats, quenda and brushtail possums

(✓ = known reservoir host; ✓ = infection has been identified in this host species;

✗ = infection has not been identified in this host species)

Giardia spp. infection identified:	Humans	Dogs	Cats	Quenda[1]	Brushtail possums[1]
G. duodenalis	✓	✓	✓	✓[2]	✓[2]
Genotype AI	✓	✓	✓	✗	✗
Genotype AII	✓	✓	✓	✗	✗
Genotype AIII	✓	✓	✓	✗	✗
G. enterica	✓	✓	✓[2]	✗	✗
Genotype BIII	✓	✓	✗	✗	✗
Genotype BIV	✓	✗	✗	✗	✗
G. canis	✗[3]	✓	✓	✓	✗
Genotype C	✗[3]	✓	✓	✓	✗
Genotype D	✗[3]	✓	✓	✗	✗
G. bovis	✗[3]	✓	✓	✓	✗
G. cati	✗[3]	✗	✓	✗	✗
'Quenda genotype'	✗	✗	✗	✓	✗
Other *Giardia* spp.	✗	✗	✗	✗	✗

[1] Based on limited available data

[2] Not differentiated to genotype level

[3] Some studies have identified these species and genotypes, but the methods of genetic characterisation are considered insufficient to be conclusive (Feng and Xiao, 2011)

1.3.3 Australian marsupial hosts shown to be susceptible to *Giardia* spp. infection

Giardia duodenalis, *G. enterica*, *G. canis* genotypes C and D, *G. bovis* and the 'quenda genotype' of *Giardia* have been isolated from faeces of various Australian marsupial hosts.

Giardia duodenalis has been isolated in faecal samples from free-ranging: kangaroos (Family Macropodidae - species not described) and western grey kangaroos (*Macropus fuliginosus*) in Western Australia (McCarthy et al., 2008; Thompson (J) et al., 2008); swamp wallabies (*Wallabia bicolor*) in Victoria (Thompson (J) et al., 2008), brush-tailed rock-wallabies (*Petrogale penicillata*) in New South Wales (Vermeulen et al., 2015), koalas (*Phascolarctos cinereus*) in South Australia (Thompson (J) et al.,

2008), brushtail possums and mountain brushtail possums (*Trichosurus cunninghami*) in Victoria (Thompson (J) et al., 2008); common planigale (*Planigale maculata*) in Western Australia (Thompson et al., 2010a); and quenda in Western Australia (Thompson et al., 2010a).

Testing of captive animals has also revealed that red kangaroos (*Macropus rufus*), parma wallabies (*Macropus parma*), tammar wallabies (*Macropus eugenii*), yellow-footed rock-wallabies (*Petrogale xanthopus)*, quokka *(Setonix brachyurus)*, long-nosed potoroos (*Potorous tridactylus*), southern hairy-nosed wombats (*Lasiorhinus latifrons)* and rufous bettongs (*Aepyprymnus rufescens)* are susceptible to infection with *G. duodenalis* (Thompson (J) et al., 2008).

Giardia enterica has been isolated from free-ranging western grey kangaroos in Western Australia (Thompson (J) et al., 2008), and free-ranging kangaroos (Family Macropodidae - species not described) in New South Wales (Ng et al., 2011). Findings from captive animals suggest that red kangaroos, parma wallabies, tammar wallabies and spotted-tailed quolls (*Dasyurus maculatus)* are also susceptible to infection with *G. enterica* (Thompson (J) et al., 2008).

Giardia canis genotype C has been isolated from free-ranging quenda in Western Australia (Thompson et al., 2010a) and kangaroos in New South Wales (Ng et al., 2011), and *G. canis* genotype D has been isolated from free-ranging kangaroos in New South Wales (Ng et al., 2011).

Giardia bovis has been isolated from faeces of free-ranging quenda in Western Australia (Thompson et al., 2010a).

The 'quenda genotype' of *Giardia* has been isolated from free-ranging quenda in Western Australia (Adams et al., 2004; Thompson et al., 2010a).

"*Giardia lamblia*" was isolated from the faeces of free-ranging brushtail possums in Queensland (Ahmed et al., 2012). It is unclear what species this represents. *Giardia* spp. infection (not differentiated to species level) has also been identified in free-ranging Bennett's wallabies (*Macropus rufogriseus*), Tasmanian pademelons (*Thylogale billardierii*), long-nosed potoroos (*Potorous tridactylus*), ringtail possums (*Pseudocheirus peregrinus*), common wombats (*Vombatus ursinus*), dasyurids (Family Dasyuridae - species not indicated) and bandicoots (*Isoodon obesulus* and / or *Perameles gunnii*) in Tasmania (Bettiol et al., 1997).

Eastern barred bandicoots (*Perameles gunnii*) have also been shown to be susceptible to *Giardia* spp. infection of human origin via experimental infection, although it is not clear what species was used (presumptively it would have been *G. duodenalis* or *G. enterica*) (Bettiol et al., 1997).

1.3.4   *Giardia* spp. detection tests for use in Australian marsupial species

No tests have been validated for use in detecting *Giardia* spp. infection in any members of the respective orders of quenda or brushtail possums (Peramelemorphia or Diprotodontia).

1.3.4.1   Centrifugal faecal flotation microscopy

Centrifugal faecal flotations are used to concentrate any *Giardia* spp. cysts present in a faecal sample on the surface of a solution into which a faecal sample is mixed. The solution used is typically zinc sulphate, sodium nitrate, sugar or magnesium sulphate. The faecal sample is mixed and centrifuged with the solution, and the surface material is then transferred to a slide and examined microscopically for the presence of *Giardia* spp. cysts (Tangtrongsup and Scorza, 2010). Cysts can be detected using light microscopy, based on morphological features (Geurden et al., 2008). Centrifugal faecal

flotation microscopy is generally only able to differentiate *Giardia* spp. infections to a genus level, as the species *G. duodenalis, G. enterica, G. canis, G. bovis, G. cati* and *G. simondi*, and the 'pinniped genotype' and 'quenda genotype' of *Giardia*, all have morphologically indistinguishable cysts.

Previous research suggests that centrifugal faecal flotation microscopy is poorly sensitive in detecting *Giardia* spp. infections in dogs and humans. In testing a single faecal sample, sensitivity estimates were 26.4% (95% CI 14.4 - 50.4%) in humans (Traub et al., 2009), 34% (95% CI 6 - 80%) in dogs (Geurden et al., 2008) and 45% (95% CI 41 - 49%) in dogs (Rishniw et al., 2010). Sensitivity estimates rose to 72% (95% CI 66 - 79%) when examining pooled samples of three faecal specimens collected from the dog within one week (Rishniw et al., 2010). In contrast, centrifugal faecal flotation sensitivity was estimated at 85.3% (95% CI 68.9 - 95.1%) in cats, when examining a single faecal specimen (Mekaru et al., 2007).

Centrifugal faecal flotation microscopy is highly specific in humans, dogs and cats, at 92% or greater (Mekaru et al., 2007; Geurden et al., 2008; Traub et al., 2009; Rishniw et al., 2010). However, pooling three faecal samples from the same dog within one week has been show to substantially reduce specificity (Rishniw et al., 2010).

The accuracy of centrifugal faecal flotation microscopy may be influenced by the experience of the microscopist, as well as the particular faecal flotation protocol employed (Gates and Nolan, 2009).

1.3.4.2   Immunofluorescence microscopy of faeces

In immunofluorescence microscopy, fluorescein-labelled monoclonal antibodies directed against specific *Giardia* spp. antigens are added to the processed faecal sample. They bind to *Giardia* spp. cysts, which are then clearly identifiable by

fluorescence microscopy in fluorescing a bright apple-green. As for centrifugal faecal flotation microscopy, immunofluorescence microscopy of faeces is generally only able to differentiate *Giardia* spp. to genus level, as cysts of *G. duodenalis, G. enterica, G. canis, G. bovis, G. cati* and *G. simondi*, and the 'pinniped genotype' and 'quenda genotype' of *Giardia*, are morphologically indistinguishable.

Immunofluorescence has a reported 90 - 100% sensitivity and 94 - 100% specificity in detecting *Giardia* spp. cysts in human and dog faecal samples (Garcia et al., 1992; Alles et al., 1995; Azia et al., 2001, Geurden et al., 2008; Rishniw et al., 2010). However, whilst Traub et al. (2009) found immunofluorescence microscopy highly specific in humans, the point estimate for sensitivity was lower, at 61.8% (95% CI 40.8 - 99.1%).

1.3.4.3 Faecal antigen detection tests (immunoassays)

Immunoassays, both enzymatic and non-enzymatic, are used to detect *Giardia* spp. antigens in faeces. They do not differentiate between *Giardia* species. Sensitivity estimates of various ELISAs range from 45 - 95.12% in a single dog, cat or human faecal sample, with specificity estimates of 92 - 100% (Mekaru et al., 2007; Geurden et al., 2008; Rishniw et al., 2010; Elsafi et al., 2013; Kamel et al., 2013).

1.3.4.4 Molecular diagnostic tools

Molecular tools have been developed to differentiate *Giardia* to species level. These include polymerase chain reaction (PCR), restriction fragment length polymorphisms (RFLP) and sequencing. These techniques involve the amplification of certain gene(s) of *Giardia* DNA present in a faecal sample, which enables identification of *Giardia* spp. in the sample. Differentiation to genotype level can be made via sequencing (Wielinga and Thompson, 2007; Feng and Xiao, 2011). Four of the most common sites for genotyping in research studies include the 18S ribosomal RNA (18s rRNA), β-giardin

(*bg*), glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) loci (Caccio et al., 2008; Sprong et al., 2009; Feng and Xiao, 2011).

The 18S rRNA locus is frequently used to identify *Giardia* spp. infection in a sample, due to several advantages. Firstly, there are an estimated 60 copies of the 18S rRNA repeat per genome, compared to one or a low number in other loci, which entails a greater success rate in amplifying the gene, and thus relatively high test sensitivity (Wielinga and Thompson, 2007). Secondly, for the same reason, it is more likely to detect mixed infections in a single test, compared to using a low copy gene locus (Wielinga and Thompson, 2007). Thirdly, its comparatively low substitution rate gives this locus higher specificity, compared to other loci (Caccio and Ryan, 2008). The more conserved regions of the locus are only able to differentiate between certain *Giardia* species, while the variable 5'3' ends of the locus can be used for differentiating a larger range of *Giardia* species. The choice of primer sets is an important consideration - if they are too small, species will not necessarily be differentiated accurately. This has been identified as a problem in previous molecular studies of *Giardia* (Feng and Xiao, 2011). As the 18S rRNA locus has limited intra-species variation, it is not suitable for differentiating closely related species and genotypes of *Giardia* (Caccio et al., 2008; Sprong et al., 2009; Lebbad et al., 2010).

Whilst the three other commonly used loci have fewer copies per genome, they are sufficiently variable to enable differentiation of closely related species and genotypes of *Giardia*. Most primers amplify a large proportion of these loci (60% of the *tpi* and *bg* loci, and 40 - 60% of the *gdh* locus), so genotype results are considered reliable (Feng and Xiao, 2011). However, identification of *Giardia* spp. may vary between loci (Sprong et al., 2009; Ballweber et al., 2010; Feng and Xiao, 2011). This is believed to be attributable to the presence of mixed infections, or allelic sequence heterozygosity between the two nuclei in each *Giardia* cyst (due to genetic recombinations and introgression, post translational modifications, or the presence of ancestral

polymorphisms at the locus targeted) (Weilinga and Thompson, 2007; Caccio and Ryan, 2008; Sprong et al., 2009; Ballweber et al., 2010). Therefore, using sequence analysis at a single locus to differentiate *Giardia* spp. infection to a species or genotype level may be a source of misclassification bias.

Experimentally, sensitivity of detection has been shown to remain high with low numbers of cysts present in a sample (Miller and Sterling, 2007). In single human faecal samples, various PCR techniques have estimated sensitivities of 97.2 - 100% (Verweij et al., 2003; Traub et al., 2009; Calderaro et al., 2010; Elsafi et al., 2013) and specificities of 56.2 - 100% (Verweij et al., 2003; Traub et al., 2009; Calderaro et al., 2010; Elsafi et al., 2013). However, sensitivity and specificity estimates are likely to be substantially influenced by the choice of loci included in the case definition for infection (as demonstrated in Beck et al., 2011a,b; McDowall et al., 2011; Sommer et al., 2015) and the inclusion (or not) of sequencing in the case definition (as demonstrated in Calderaro et al., 2010).

1.3.4.5   Choice of detection test for *Giardia* spp. infection in epidemiological studies of wildlife

As previously noted, no *Giardia* spp. detection tests have been validated in quenda and brushtail possums - nor in any other species of Australian marsupial. However, findings of Thompson (J) et al. (2008) and Thompson et al. (2010a) suggest that neither zinc sulphate centrifugal faecal flotations nor PCR and sequencing are 100% sensitive in the detection of *Giardia* spp. in faecal samples of Australian marsupial species.

Validation of the detection test or test protocol used in future epidemiological studies of Australian marsupial species is required to ensure validity in interpretation of epidemiological data. A validated protocol of high accuracy (both high sensitivity and high specificity) is ideal, but factors such as cost of testing, and additional information gained in undertaking a particular test, are also of consideration. A validated protocol of

sub-optimal accuracy is acceptable, in that validation at least enables estimation of the likely impact of misclassification bias on results.

Faecal microscopy and faecal antigen detection tests are inadequate as a sole *Giardia* spp. parasite detection test in epidemiological studies, as these tests cannot identify the species of *Giardia* present. However, such tests, if highly sensitive, may remain of use as a screening test, to restrict costly molecular characterisation to *Giardia* spp. positive samples only. Faecal microscopy is also of value in quantifying the amount of cyst shedding, and identifying concurrent gastrointestinal parasitic infections.

1.3.5 Impacts of *Giardia* spp. infections on marsupial health

There are little data available to provide insights as to the impact of *Giardia* spp. infections on wildlife populations (Appelbee et al., 2005; Thompson et al., 2010b). No published studies specifically investigating the clinical effects of *Giardia* spp. infections in quenda are available. A small experimental study was performed on eastern barred bandicoots (*Perameles gunnii*) - a species of the same taxonomic family as quenda (Peramelidae). In this study, 1 / 2 bandicoots experimentally inoculated *per os* with 1500 human-derived *Giardia* spp. cysts (species unknown) became infected. The prepatent period in this bandicoot was nine days, with at least an 11 day duration of patency (the total duration of post inoculation faecal testing was not given). Microscopy and CELISA were used to detect infection and monitor cyst shedding. The only clinical symptoms noted were "occasionally paler and looser faeces than normal" (Bettiol et al., 1997). This appears to be a subjective assessment, and as there is no indication that observer blinding was used in experimental design, observer bias may have influenced this finding. Further, the dose of infection was large (1500 cysts), compared to established human minimum infective doses, which may not reflect typical doses of infection to free-ranging bandicoots; this may limit the applicability of the study findings to naturally-acquired infections.

As for quenda, no published studies have specifically investigated clinical symptoms associated with *Giardia* spp. infections in brushtail possums. As part of a wider study, Thompson (J) et al. (2008) identified *Giardia* spp. infection via PCR in 21 / 84 (25%) possums (Suborder Phalangeriformes - including 68 brushtail possums) and found all faecal samples to be of normal consistency. However, the potential influences of selection bias and a lack of temporality in these observations are substantial limitations.

Mixed *Giardia* spp. infections have not yet been identified in quenda or brushtail possums, but this may be due to the limited investigations undertaken thus far. The relative virulence and pathogenicity of the host-adapted 'quenda genotype' of *Giardia* and other *Giardia* spp. infections in quenda or brushtail possums is unknown. It is not known what impact other *Giardia* spp. have on the circulation of the host-adapted 'quenda genotype' in quenda - whether other *Giardia* spp. tend to co-exist with, outcompete, or be outcompeted by the 'quenda genotype'. No host-adapted *Giardia* sp. has been identified infecting brushtail possums. It is also unknown as to whether concurrent infection with multiple species or genotypes has any impact on clinical disease in quenda or possums.

1.3.6 *Giardia* spp. in Australian marsupial species: prevalence and risk factors

It is particularly difficult to estimate prevalence of infection in wildlife populations in a manner that is robust to selection bias (i.e. measured by proportionate sampling). Applying proportionate sampling strategies to marsupial populations over large target population areas is generally not realistically achievable - in addition to the typically limited knowledge of the underlying population distributions, there are obvious logistical impracticalities in randomly sampling free-ranging populations. Therefore, all studies reviewed have involved a form of non-proportionate sampling, which may have resulted in sampling bias substantially influencing the data. Additionally, the data collection

methods required in sampling free-ranging marsupials (e.g. trapping) are possibly a further source of selection bias. This is an important limitation to be considered when extrapolating analytically from these study prevalences to make population level inferences. Further, a lack of precision in prevalence estimates, due to relatively small sample sizes, is a common limitation, as is a lack of validation of the parasite detection tests used to differentiate infected animals from non-infected animals.

Prevalences of *Giardia* spp. infection in quenda and other bandicoots (Family Peramelidae) and brushtail possums and other possums (Suborder Phalangeriformes) are summarised in Tables 1.3 and 1.4.

Table 1.3 *Giardia* spp. identified in quenda (syn. southern brown bandicoots, *Isoodon obesulus*) and other Australian bandicoots (Family Peramelidae)

Species, study site[1] (study)	Sampling timeframe	Parasite detection test	No. infected / no. tested (%; 95% CI[2])	Species / genotype identified
Quenda, near Manjimup, WA (Adams et al., 2004)	Unspecified	Microscopy (PCR at 18S rRNA & ef1α loci with sequencing)[3]	1 / 72 (1.4%; 0.3 - 7.4%)	1 x 'quenda genotype'
Quenda, Batalling, WA (Adams et al., 2004)	Unspecified	Microscopy	0 / 4 (0%; 0 - 52.2%)	-
Quenda, Perth, WA (Adams et al., 2004)	Unspecified	Microscopy	0 / 1 (0%; 0 - 84.2%)	-
Quenda, WA (Thompson (J) et al., 2008)	Feb 2006 - March 2007	PCR (18S rRNA locus)	0 / 3 (0%; 0 - 60.2%)	-
Quenda, Perth, WA (Thompson et al., 2010a)	Unspecified	Microscopy and PCR (β-giardin and 18S rRNA loci with sequencing)	9 / 14 (64.3%; 38.4 - 83.7%)	5 x 'quenda genotype' 1 x *G. duodenalis* 3 x *G. canis*
Quenda, Upper Warren, WA (Thompson et al., 2010a)	Unspecified	Microscopy and PCR (β-giardin & 18S rRNA loci with sequencing)	2 / 9 (22.2%; 6.7 - 55.6%)	1 x 'quenda' genotype[4]
Quenda, Barrow Island, WA (Thompson et al., 2010a)	Unspecified	Microscopy and PCR (β-giardin & 18S rRNA loci with sequencing)	1 / 28 (3.6%; 0.8 - 17.8%)	1 x *G. bovis*
Quenda, Julimar, WA (Thompson et al., 2010a)	Unspecified	Microscopy and PCR (β-giardin & 18S rRNA loci with sequencing)	2 / 4 (3.6%; 14.7 - 85.3%)	-[5]
Southern brown bandicoots & eastern barred bandicoots (*Perameles gunnii*)[6], Tasmania (Bettiol et al., 1997)	1993 – 1995	Microscopy and ELISA[3]	16 / 26 (62%; 42.4 - 77.6%)	-
Western barred bandicoots (*P. bougainville*), Shark Bay, WA (Adams, 2003)	1999 - 2001	Microscopy	0 / 35 (0%; 0 - 9.7%)	-
Western barred bandicoots, WA[7] (Bennett, 2008)	2005 - 2007	Microscopy and PCR (18S rRNA locus)	0 / 22 (0%; 0 - 14.8%)	-

[1] WA = Western Australia.

[2] Calculated by thesis author using Jeffrey's 95% CI methods.

[3] Unclear if tested in parallel or series.

[4] One sample was not successfully sequenced.

[5] Two samples not successfully sequenced.

[6] Results for southern brown bandicoots and eastern barred bandicoots were pooled.

[7] Dryandra, Heirisson Prong, Bernier Island, Dorre Island.

Table 1.4 *Giardia* spp. identified in free-ranging brushtail possums (*Trichosurus vulpecula)* and other possum species (Suborder Phalangeriformes) in Australia

Species, study location[1] (study)	Sampling timeframe	Parasite detection test	No. infected / no. tested (%; 95% CI[2])	Species / subspecies identified
Brushtail possums, Manjimup & Batalling, WA (Adams, 2003)	1999 - 2001	Microscopy	0 / 249 (0%; 0 - 1.5%)	-
Brushtail possums, Lake Magenta, WA (Thompson et al., 2010a)	Unspecified	Microscopy	0 / 7 (0%; 0 - 36.9%)	-
Brushtail possums, Upper Warren, WA (Thompson et al., 2010a)	Unspecified	Microscopy	0 / 101 (0%; 0 - 3.6%)	-
Brushtail possums, Barrow Island, WA (Thompson et al., 2010a)	Unspecified	Microscopy	0 / 17 (0%; 0 - 18.5%)	-
Brushtail possums, Sydney, NSW (Cox et al., 2005)	April - May 2002	Microscopy	0 / 2 (0%; 0 - 70.8%)	-
Brushtail possums, Victoria (Thompson (J) et al., 2008)	February 2006 - March 2007	PCR (18S rRNA locus, with sequencing)	13 / 48 (27.1%; 16.6 - 41.1%)	11 x *G. duodenalis*
Mountain brushtail possums (*Trichosurus cunninghami*), Victoria (Thompson (J) et al., 2008)	February 2006 - March 2007	PCR (18S rRNA locus, with sequencing)	7 / 32 (21.9%; 11.1 - 38.9%)	7 x *G. duodenalis*
Brushtail possums & ringtail possums (*Pseudocheirus peregrinus*)[3], Tas (Bettiol et al., 1997)	1993 – 1995	Microscopy and ELISA[4]	18 / 93 (19.4%; 12.6 - 28.6%)	-
Ringtail possums, Australia (Thompson (J) et al., 2008)	February 2006 - March 2007	PCR (18S rRNA locus)	1 / 2 (50%; 9.4 - 90.6%)	-
Western ringtail possums (*Pseudocheirus occidentalis*), WA (Thompson (J) et al., 2008)	February 2006 - March 2007	PCR (18S rRNA locus)	0 / 1 (0%; 0 - 84.2%)	-
Sugar glider (*Petaurus breviceps*), Australia (Thompson (J) et al., 2008)	February 2006 - March 2007	PCR (18S rRNA locus)	0 / 2 (0%; 0 - 70.8%)	-

[1] WA = Western Australia; NSW = New South Wales; Tas = Tasmania.

[2] Calculated by thesis author using Jeffrey's 95% CI methods.

[3] Proportions of brushtail possums and common ringtail possums amongst the infected and total sample size not described.

[4] Unclear how tests were combined for case definition.

1.3.6.1 Risk factors for infection

In a study involving 209 captive and 212 free-ranging Australian marsupial faecal samples (including three quenda samples and 48 brushtail possum samples), Thompson (J) et al. (2008) found no associations between *Giardia* infection and host sex, the season in which the sample was obtained, or rainfall. Mean temperature was lower in areas where a positive faecal sample was found, however the heterogeneous species composition of the study sample (which encompassed three suborders of marsupials) compromises the validity of analytical extrapolations. Additionally, confounding by captivity was not considered in statistical analyses, and this may have influenced the observed association between mean temperature and the presence of *Giardia* spp. Further, a lack of power cannot be ruled out as an influence on the lack of association between *Giardia* spp. and sex, as host sex was known for only 56 samples.

1.4   *Toxoplasma gondii* infection in Australian marsupial species

1.4.1   Introduction

Australia is home to a vast array of endemic marsupial species (Superorder Australidelphia). Of an estimated 162 marsupial species present at the time of European settlement in 1788, twelve species have since become extinct, and many others have suffered major contraction of distribution and substantial to severe population declines (Burbidge et al., 2009; Woinarski, 2015). The primary drivers of these population declines are believed to be: anthropogenic habitat loss; habitat destruction associated with climate change, altered fire regimes and the introduction of competing feral herbivores and exotic weeds; and predation by introduced feral species, particularly foxes and cats (Dickman, 1996; Fisher et al., 2003; McKenzie et al., 2007; Burbidge et al., 2009; Saunders et al., 2010).

There has also been speculation as to the role of introduced infectious disease as a contributing factor to population declines of Australian marsupials (e.g. Freeland, 1994; Abbott, 2006; Thompson et al., 2010b).*Toxoplasma gondii* is arguably the most broadly implicated infection, prompted by reports of cases and outbreaks of severe clinical toxoplasmosis in captive populations of Australian marsupial species. From the published literature, there appears to be a perception that marsupials are particularly susceptible to infection with *T. gondii* and to toxoplasmosis. For example: "Toxoplasmosis is a significant disease of Australian marsupials commonly causing mortality in captive and free-ranging populations…" (Obendorf et al., 1996); "Marsupials and new world monkeys are among the most susceptible animals for developing the clinical disease toxoplasmosis…" (Skerratt et al., 1997); "Australasian marsupials, especially wallabies, are highly susceptible to acute toxoplasmosis" (Dubey and Crutchley, 2008); "Australian marsupials are among the most susceptible hosts for *Toxoplasma gondii*…" (Parameswaran et al., 2009a); "*T. gondii* is of concern for

Australian native marsupials, which appear to be particularly susceptible to acute infection…" (Hollings et al., 2013).

In this section, the evidence for these assertions is examined, by reviewing estimates of the frequency of *T. gondii* infection in free-ranging Australian marsupial populations, and then reviewing the extent to which the infections are associated with acute toxoplasmosis or with other effects (such as behavioural changes and reduced reproductive success) that may threaten population viability.

1.4.1.1 *Toxoplasma gondii* infection and toxoplasmosis

Toxoplasma gondii is a protozoan parasite, which can infect a wide range of endothermic vertebrates. Cats (Felidae) are the definitive host - infected cats shed environmentally resistant oocysts in their faeces. Oocysts become infective in the environment, and if ingested can infect both intermediate hosts (including Australian marsupial species) and other definitive hosts. Following ingestion, sporozoites excyst from oocysts, invade the gut epithelium and transform into tachyzoites. Tachyzoites multiply asexually and may colonise many host tissues, evoking a strong immune response. Tachyzoites differentiate into bradyzoites, which produce tissue cysts that are resistant to the immune response. Bradyzoites may be transmitted to a definitive host, or another intermediate host, upon ingestion of infected tissues. In addition, these hosts may also be infected via vertical transmission from infected mother to foetus / suckling young (Dubey, 1998; Dubey, 2010).

In most intermediate host species, including people, *T. gondii* infection tends to be subclinical; toxoplasmosis (clinical disease caused by *T. gondii* infection) is usually associated with complicating factors such as immunosuppression (Montoya and Liesenfeld, 2004; Dubey, 2010). Clinical toxoplasmosis may follow recent infection with *T. gondii*, or result from a recrudescent infection. Recrudescence may be prompted by concurrent illness or immunosuppression (Ruskin and Remington, 1976; Lappin et al., 1991; Nicoll et al., 1997).

1.4.2 The frequency of *Toxoplasma gondii* infection in free-ranging populations of Australian marsupial species

No published studies have investigated the incidence of *T. gondii* infection in free-ranging populations of Australian marsupials**.** Surveys have provided estimates infection prevalence and seroprevalence; these are summarised in Tables 1.5 and 1.6.

1.4.2.1 *Toxoplasma gondii* infection surveys undertaken in free-ranging populations of Australian marsupials

Evidence of *T. gondii* infection has been found in free-ranging populations of red kangaroos (*Macropus rufus*), western grey kangaroos (*M. fuliginosus*), common wallaroos (*M. robustus*) and woylies (*Bettongia penicillata*) (Table 1.5). Findings suggestive of *T. gondii* infection (histopathological evidence without confirmatory testing) have also been obtained from long nosed bandicoots *(Perameles nasuta)*, eastern barred bandicoots (*P. gunnii*), southern brown bandicoots / quenda (*Isoodon obesulus*), quokka (*Setonix brachyurus*), brushtail possums (*Trichosurus vulpecula*), brush-tailed phascogales (*Phascogale tapoatafa*) and kowari (*Dasyuroides byrnie*) (Table 1.5)*.*

Table 1.5 *Toxoplasma gondii* infection surveys undertaken in free-ranging populations of Australian marsupial species

Except where noted, no prior clinical suspicion of toxoplasmosis existed in sampled animals.

Species	Study location (study)	Sampling timeframe	Parasite detection test[1]	No. infected / no. tested (%; 95% CI[2])
Eastern grey kangaroo *(Macropus giganteus)*	Roma, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 4 (0%; 0 - 52.2%)
Eastern grey kangaroo	Blackall, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 3 (0%; 0 - 60.2%)
Western grey kangaroo *(M. fuliginosus)*	Menzies, Western Australia (Pan et al., 2012)	2008	PCR (heart, liver, lung, spleen, diaphragm)	5 / 5 (100%; 54.1 - 99.6%)
Western grey kangaroo- adults	Perth, Western Australia (Parameswaran et al., 2010)	Not specified	PCR (brain and tongue)	9 / 12 (75%; 46.2 - 90.9%)
Western grey kangaroo- pouch young	Perth, Western Australia (Parameswaran et al., 2010)	Not specified	PCR (brain, heart, skeletal muscle, liver, lung, small intestine, kidney and spleen)	2 / 17 (11.8%; 3.6 - 34.7%)
Common wallaroo *(M. robustus)*	Blackall, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 5 (0%; 0 - 45.9%)
Common wallaroo	Kynuna, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 3 (0%; 0 - 60.2%)
Common wallaroo	Menzies, Western Australia (Pan et al., 2012)	2008	PCR (heart, liver, lung, spleen, diaphragm)	5 / 5 (100%; 54.1 - 100%)
Red kangaroo *(M. rufus)*	Blackall, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 5 (0%; 0 - 45.9%)

Species	Study location (study)	Sampling timeframe	Parasite detection test[1]	No. infected / no. tested (%; 95% CI[2])
Red kangaroo	Longreach, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 6 (0%; 0 - 41.0%)
Red kangaroo	Menzies, Western Australia (Pan et al., 2012)	2008	PCR (heart, liver, lung, spleen, diaphragm)	6 / 6 (100%; 59.0 - 100%)
Woylie[3] *(Bettongia penicillata)*	Manjimup, Western Australia (Parameswaran et al., 2010)	Not specified	PCR (brain and heart)	2 / 4 (50%; 14.7 - 85.3%)
Long-nosed potoroo *(Potorous tridactylus)*	Winkleigh, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 1 (0%; 0 - 84.2%)
Quokka *(Setonix brachyurus)*	Rottnest Island, Western Australia (Gibb et al., 1966)	Nov 1961	Histopathology (left lateral femoral muscle biopsy)	4 / 92 (4.3%; 1.8 - 10.6%)
		Nov 1963		14 / 20 (70%; 47.8 - 85.4%)
		Jan 1964		1 / 20 (5%; 1.2 - 23.8%)
		Feb 1964		6 / 18 (33.3%; 16.3 - 56.6%)
Quokka[4]	Rottnest Island, Western Australia (Gibb et al., 1966)	Nov 1963 - Feb 1964	Mouse bioassay (suspension of host skeletal muscle, +/- liver, brain and heart, injected IP and combined IP & IC; impression smears +/- histopathological examination of mice) 13-day old chick embryo inoculation (suspension of host skeletal muscle, cardiac muscle and brain)	2 / 28 (7.1%; 2.2 - 22.8%)
Brushtail possum *(Trichosurus vulpecula)*	Brisbane, Queensland (Cook and Pope, 1959)	Not specified	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	2 / 7 (28.6%; 8.5 - 65.1%)

Species	Study location (study)	Sampling timeframe	Parasite detection test[1]	No. infected / no. tested (%; 95% CI[2])
Brushtail possum	Dalby, Queensland (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain or pooled organs, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 3 (0%; 0 - 60.2%)
Brushtail possum	Cressy, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain or pooled organs, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 9 (0%; 0 - 30.8%)
Western Ringtail possum (*Pseudocheirus occidentalis*)[5]	Western Australia (Parameswaran, 2008)	Not specified	PCR (heart of one possum, skeletal muscle of another)	0 / 2 (0%; 0 - 70.8%)
Brush-tailed phascogale (*Phascogale tapoatafa)*	Burleigh, Queensland (Cook and Pope, 1959)	Not specified	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	1 / 1 (100%; 15.8 - 98.7%)
Eastern pygmy possum (*Cercartetus nanus*)	Wilmot, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 1 (0%; 0 - 84.2%)
Southern brown bandicoot (*Isoodon obesulus*)	Brisbane & Innisfail, Queensland (Cook and Pope, 1959)	Not specified	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	5 / 10 (50%; 23.4 - 76.6%)
Southern brown bandicoot	Brisbane, Queensland (Pope et al., 1957a)	August 1955 – February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	16 / 38 (42.1%; 27.8 - 57.9%)

Species	Study location (study)	Sampling timeframe	Parasite detection test[1]	No. infected / no. tested (%; 95% CI[2])
Southern brown bandicoot	Mt Glorius, Queensland (Pope et al., 1957a)	August 1955 – February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	0 / 2 (0%; 0 - 70.8%)
Southern brown bandicoot	North Queensland (Pope et al., 1957a)	August 1955 – February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	1 / 3 (33.3%; 6.8 - 80.6%)
Southern brown bandicoot	Brisbane, Queensland (Pope et al., 1957b)	1951	Histopathology (brain, lungs, liver, spleen, kidney, retroperitoneal lymph node); Mouse and guinea pig bioassays (suspension of host liver, spleen and brain injected IP and IC; histopathological examination of mouse brain, heart, lungs, spleen, skeletal muscle, kidneys, pancreas, blood)	1 / 1 (100%; 15.8 - 98.7%)
Long-nosed bandicoot (*Perameles nasuta*)	Mt Glorius, Queensland (Pope et al., 1957a)	August 1955 – February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	1 / 2 (50%; 9.4 - 90.6%)
Long-nosed bandicoot	Northern Queensland (Pope et al., 1957a)	August 1955 – February 1956	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	2 / 4 (50%; 14.7 - 85.3%)

Species	Study location (study)	Sampling timeframe	Parasite detection test[1]	No. infected / no. tested (%; 95% CI[2])
Eastern barred bandicoot (*P. gunnii*)	Longford, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 1 (0%; 0 - 84.2%)
Eastern barred bandicoot[6]	Tasmania (Obendorf et al., 1996)	July 1992 - March 1995	Histopathology (brain, heart, lung and skeletal muscle) with serology	7 / 8 (87.5%; 51.8 - 97.2%)
Greater bilby (*Macrotis lagotis*)	Birdsville, Queensland (Cook and Pope, 1959)	Not specified	Mouse bioassay (host brain suspension inoculated IC or host liver spleen, kidney and lung suspension inoculated IP; if mouse necropsy was suggestive of *T. gondii* infection, contact smear of brain performed)	0 / 1 (0%; 0 - 84.2%)
Tasmanian devil (*Sarcophilus harrisii*)	Gladstone, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 3 (0%; 0 - 60.2%)
Eastern quoll (*Dasyurus viverrinus)*	Gladstone, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 7 (0%; 0 - 36.9%)
Spotted-tailed quoll (*D. maculatus*)	Wilmot, Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 1 (0%; 0 - 84.2%)
Northern quoll[7] (*D. hallucatus*)	Kakadu National Park, Northern Territory (Oakwood and Pritchard, 1999)	1993 - 1995	Histopathology (brain, heart, lungs, diaphragm, "gut", liver, tongue, hind leg muscle)	0 / 28 (0%; 0 - 11.9%)
Kowari (*Dasyuroides byrnie)*	Queensland (Attwood et al., 1975)	Not specified	Histopathology (brain, spinal cord, heart, lung, kidney, adrenal gland, stomach, "gut", spleen, pancreas, parapancreatic node)	4 / 17 (23.5%; 9.7 - 47.6%)

Species	Study location (study)	Sampling timeframe	Parasite detection test[1]	No. infected / no. tested (%; 95% CI[2])
Dusky Antechinus (*Antechinus swainsonii*)	Tasmania (Smith and Munday, 1965)	Not specified	Mouse bioassay (suspension of host brain, +/- liver, lung & spleen, injected SC; mouse brain emulsified and examined for *T. gondii* cysts)	0 / 1 (0%; 0 - 84.2%)
Brown Antechinus *(A. stuartii)*	Not specified (Attwood et al., 1975)	Not specified	Histopathology (brain, spinal cord, heart, lung, kidney, adrenal gland, stomach, "gut", spleen, pancreas, parapancreatic node)	0 / 3 (0%; 0 - 60.2%)
White-footed dunnart *(Sminthopsis leucopus)*	Not specified (Attwood et al., 1975)	Not specified	Histopathology (brain, spinal cord, heart, lung, kidney, adrenal gland, stomach, "gut", spleen, pancreas, parapancreatic node)	0 / 7 (0%; 0 - 36.9%)

[1] Regarding mouse bioassays – SC = subcutaneous inoculation, IP = intraperitoneal inoculation, IC = intracerebral inoculation.

[2] Calculated by review author using Jeffrey's 95% confidence interval.

[3] One (infected) woylie exhibited neurological symptoms prior to death.

[4] Survey of wild quokka with serological or histological evidence of *T. gondii* infection.

[5] Wild possums found with neurological symptoms.

[6] One bandicoot obtained via trap death (previously identified as seropositive for *T. gondii*); the origin of the other bandicoots is unclear.

[7] Study subjects obtained via roadkill.

Prevalence estimates are all limited by uncertain external validity, due to the use of non-proportionate sampling methods: reliance on culled animals, road kill or trapping for study subjects may entail selection bias. Surveys involving small sample sizes have low power to detect the presence of infection, and marked imprecision in prevalence estimates (Table 1.5). Commonly, the use of parasite detection methodology that is known to be of poor sensitivity and / or specificity in other species leaves apparent prevalence estimates subject to misclassification bias.

In marsupial surveys, the most commonly used parasite detection test has been the mouse bioassay, which lacks sensitivity (Piergili Fioretti, 2004). None of the surveys of Australian marsupials using this technique also used immunohistochemistry or PCR to confirm identification of *T. gondii* bradyzoites. Thus, the specificity of the mouse

bioassay may be compromised, as *T. gondii* bradyzoites can appear very similar to those of *Neospora caninum* under light microscopy (Dubey et al., 2009). As with the mouse bioassay, sample inoculation into cell culture (in this case 13-day old chick embryos (Table 1.5) lacks sensitivity, often because of laboratory error (Piergili Fioretti, 2004).

Histopathological examination of host tissues, without confirmatory immunohistochemistry or PCR, was used in a number of marsupial surveys (Table 1.5). However, this is also an insensitive screening tool, particularly in identifying low burden *T. gondii* infections (Piergili Fioretti, 2004). Specificity of these results might also be compromised by misidentification of other protozoan parasites as *T. gondii* (Dubey et al., 2009).

PCR amplification of *T. gondii* DNA in tissue samples is generally considered a sensitive indicator of infection (Burg et al., 1989; Su et al., 2010). PCR has only been used in two surveys of Australian marsupials, which collectively sampled four species (Parameswaran et al., 2010; Pan et al., 2012). A high proportion these animals tested positive for *T. gondii* by this methodology. Though these findings are limited by small sample sizes, they sharply contrast to collective findings of similar species surveyed histopathologically, where infection was rarely identified (Table 1.5). As the latter studies were based on different species in different locations, the results may simply reflect heterogeneity in the distribution of infection. However, such discordant results do lend support to the hypothesis that histopathological surveys are insensitive in estimating prevalence of infection in marsupial populations showing no clinical signs of toxoplasmosis. PCR techniques have been demonstrated to have high specificity (Burg et al., 1989): given the use of appropriate negative controls and sequencing in both studies, it seems unlikely that false positives would have substantially influenced the findings of the PCR surveys.

1.4.2.2 *Toxoplasma gondii* **exposure in free-ranging populations of Australian marsupials**

Toxoplasma gondii serosurveys have found *T. gondii* antibodies in populations of western grey kangaroos, eastern grey kangaroos (*Macropus giganteus*), Bennett's wallabies *(M. rufogriseus),* bridled nailtail wallabies *(Onychogalea fraenata)*, Tasmanian pademelons *(Thylogale billardierii),* brush tailed rock wallabies (*Petrogale penicillata),* quokkas*,* woylies, Tasmanian devils (*Sarcophilus harisii*), common wombats *(Vombatus ursinus),* eastern quolls (*Dasyurus viverrinus*), spotted-tail quolls (*D. maculatus*), chuditch (or western quolls, *D. geoffroi),* brushtail possums, western ringtail possums (*Pseudocheirus occidentalis*), southern brown bandicoots*,* eastern barred bandicoots, long-nosed bandicoots and bilbies *(Macrotis lagotis)* (Table 1.6).

Table 1.6 *Toxoplasma gondii* seroprevalence surveys undertaken in free-ranging populations of Australian marsupial species

Except where noted, animals were sampled or taken from the wild via trapping or culls, and no prior clinical suspicion of toxoplasmosis existed in sampled animals.

Species	Study location (study)	Sampling timeframe	Serological Test (restrictions on antibody type detected, if any) *Cut off for seropositivity*	No. seropositive / no. tested (%; 95% CI)
Western grey kangaroo (*Macropus fuliginosus*)	Perth, Western Australia (Parameswaran, 2008; Parameswaran et al., 2009a)	May 2005 - May 2007	ELISA (IgG only) *Optical density (OD) ≥ 0.636[1]*	34 / 219 (15.5%; 10.7 - 20.3%)
Eastern grey kangaroo (*M. giganteus*)	Roma, Queensland (Parameswaran, 2008)	2004 - 2005	ELISA (IgG only) *OD ≥ 0.636[1]*	0 / 112 (0%; 0 - 3.2%[2])
Eastern grey kangaroo	Sydney, New South Wales (Parameswaran, 2008)	May 2006	ELISA (IgG only) *OD ≥ 0.636[1]*	2 / 65 (3.1%; 0 - 7.3%)
Macropus spp.[3]	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥ 1:8[1]*	0 / 31[4] (36.7%; 0 - 10.9%[2])
Bennett's wallaby (*M. rufogriseus rufogriseus*)	Tasmania (Munday, 1972)	Not specified	Sabin-Feldman dye test *Titre ≥ 1:16[1]*	0 / 1 (0%; 0 - 84.2%[2])
Bennett's wallaby	Tasmania[5] (Johnson et al., 1988)	Not specified	ELISA (IgG only) *OD ≥ 0.25[1]*	5 / 151 (3.3%; 1.5 - 7.5%[2])
Bennett's wallaby[6]	Tasmania[3] (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:64[1]*	2 / 25 (8.0%; 2.4 - 25.1%[2])
Tasmania pademelon (*Thylogale billardierii*)	Tasmania (Munday, 1972)	Not specified	Sabin-Feldman dye test *Titre ≥ 1:16[1]*	3 / 7 (42.9%; 15.7 - 75.5%[2])
Tasmanian pademelon	Tasmania[6] (Johnson et al., 1988)	Not specified	ELISA (IgG only) *OD ≥ 0.25[1]*	15 / 85 (17.7%; 11.0 - 27.1%[2])
Tasmanian pademelon[6]	Tasmania (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:64[1]*	28 / 228 (12.3%; 8.6 - 17.2%[2])
Bridled nailtail wallaby (*Onychogalea fraenata*)	Taunton National Park, Queensland (Turni and Smales, 2001)	1996	Latex agglutination test	6 / 39 (15.4%; 7.3 - 29.8%[2])
Black footed rock wallaby (*Petrogale lateralis*)	South Western Australia (Jakob-Hoff and Dunsmore, 1983)	1979	Indirect haem-agglutination inhibition test	0 / 26 (0%; 0 - 12.8%[2])
Brush tailed rock wallaby (*P. penicillata*)	South east Queensland (Barnes et al., 2010)	July 2004 - August 2005	Modified agglutination test (not IgM) *Titre ≥ 1:40[1]*	3 / 64 (4.7%; 1.7 - 12.9%[2])

Species	Study location (study)	Sampling timeframe	Serological Test (restrictions on antibody type detected, if any) *Cut off for seropositivity*	No. seropositive / no. tested (%; 95% CI)
Banded hare wallabies (*Lagostrophus fasciatus)*	Faure Island Sanctuary, Western Australia (Parameswaran, 2008)	April 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	0 / 5 (0%; 0 - 45.9%[2])
Spectacled hare wallabies (*L. conspicillatus)*	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	0 / 3 (0%; 0 - 60.2%[2])
Quokka (*Setonix brachyurus)*	Rottnest, Western Australia (Gibb et al., 1966)	1964	Sabin-Feldman dye test *Titre > 1:8*[7]	13 / 37 (35.1%; 21.8- 51.4%[2])
Burrowing bettong / boodie (*Bettongia lesueur)*	Faure Island Sanctuary, Western Australia (Parameswaran, 2008)	April 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	0 / 28 (0%; 0 - 11.9%[2])
Burrowing bettong / boodie	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	0 / 14 (0%; 0 - 21.8%[2])
Brush-tailed bettong / woylie (*Bettongia penicillata)*	Upper Warren region, Western Australia (Parameswaran, 2008)	March 2006	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	9 / 153 (5.8%; 3.2 - 10.8%[2])
Brush-tailed bettong / woylie	Dryandra Nature Reserve, Western Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	0 / 12 (0%; 0 - 24.7%[2])
Brush-tailed bettong / woylie	Batalling Forest, Western Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	0 / 17 (0%; 0 - 18.5%[2])
Brush-tailed bettong / woylie	Tutanning Nature Reserve, Western Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	0 / 8 (0%; 0 - 33.6%[2])
Brush-tailed bettong / woylie	Venus Bay Island, South Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	0 / 14 (0%; 0 - 21.8%[2])
Brush-tailed bettong / woylie	St Peters Island, South Australia (Parameswaran, 2008)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	1 / 73 (1.4%; 0.3 - 7.3%[2])
Brushtail possum (*Trichosurus vulpecula)*	Sydney (Eymann et al., 2006)	Nov 2002 - April 2005	Modified agglutination test (not IgM) *Titre ≥ 1:25*[1]	9 / 135 (6.3%; 3.6 - 12.2%[2])

Species	Study location (study)	Sampling timeframe	Serological Test (restrictions on antibody type detected, if any) *Cut off for seropositivity*	No. seropositive / no. tested (%; 95% CI)
Brushtail possum	Myall Lake National Park, New South Wales (Eymann et al., 2006)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:25[1]*	0 / 7 (0%; 0 - 36.9%[2])
Brushtail possum	Taronga Zoo grounds (non captive), Sydney (Hill et al., 2008b)	Feb 2005 - May 2006	Modified agglutination test (not IgM) *Titre ≥ 1:25[1]*	6 / 126 (4.8%; 2.2 - 10.0%[2])
Brushtail possum	Blue Mountains, New South Wales (Hill et al., 2008b)	Oct 2005 & May 2006	Modified agglutination test (not IgM) *Titre ≥ 1:25[1]*	0 / 17 (0%; 0 - 18.5%[2])
Brushtail possum	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥ 1:8[7]*	1 / 7 (14.3%; 3.2 - 52.7%[2])[4]
Brushtail possum	Tasmania (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:64[1]*	0 / 14 (0%; 0 - 21.8%[2])
Brushtail possum[7]	Kangaroo Island (O'Callaghan and Moore, 1986)	March - April 1985	Indirect haem-agglutination test	0 / 30 (0%; 0 - 11.2%[2])
Brushtail possum[8]	Western Australia (Clarke, 2011)	2006 - 2008	Direct agglutination test	0 / 95 (0%; 0 - 3.8%[2])
			Modified agglutination test (not IgM)	0 / 95 (0%; 0 - 3.8%[2])
Brushtail possum	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40[1]*	0 / 6 (0%; 0 - 41.0%[2])
Ringtail possum (*Pseudocheirus convolutor*)	Tasmania (Munday, 1972)	Not specified	Sabin-Feldman dye test *Titre ≥ 1:16[1]*	0 / 3 (0%; 0 - 60.2%[2])
Western ringtail possum (*P. occidentalis*)[8]	Western Australia (Clarke, 2011)	2006 - 2008	Direct agglutination test *Titre ≥ 1:64[7]*	2 / 99 (2.0%; 0.6 - 7.0%[2])
			Modified agglutination test (not IgM)	0 / 99 (0%; 0 - 3.6%[2])
Common planigale (*Planigale maculata*)	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40[1]*	0 / 5 (0%; 0 - 45.9%[2])
Common wombat (*Vombatus ursinus*)	Southern Tablelands, New South Wales (Hartley and English, 2005)	3/8/2001 - 25/2/2002	Latex agglutination test *Titre ≥ 1:32[1]*	1 / 23 (4.35%; 1.0 - 21.1%[2])
			Direct agglutination test *Titre > 1:64[1]*	6 / 23 (26.1%; 12.6- 46.7%[2])

Species	Study location (study)	Sampling timeframe	Serological Test (restrictions on antibody type detected, if any) *Cut off for seropositivity*	No. seropositive / no. tested (%; 95% CI)
			Modified agglutination test (not IgM) *Titre > 1:64[1]*	6 / 23 (26.1%; 12.6- 46.7%[2])
Southern brown bandicoot (*Isoodon obesulus*)	southern Western Australia (Jakob-Hoff and Dunsmore, 1983)	1979	Indirect haem-agglutination inhibition test	0 / 3 (0%; 0.0 - 60.2%[2])
Southern brown bandicoot	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥ 1:8[1]*	35 / 89 (39.3%; 29.8 - 49.7%[2])[4]
Southern brown bandicoot	Brisbane (Pope et al., 1957a)	Feb 1951	Complement fixation test *Titre ≥ 1:8[7]*	1 / 1 (100%; 15.8- 98.7%[2])
Southern brown bandicoot	Brisbane, North Qld, Mt Glorius-Queensland (Pope et al., 1957a)	August 1955 – February 1956	Complement fixation test *Titre ≥ 1:8[7]*	15 / 35 (42.9%; 27.9 - 59.2%[2])[4]
Golden bandicoots (*I. auratus*)	Barrow Island, Western Australia (Parameswaran, 2008)	September 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40[1]*	0 / 11 (0%; 0 - 26.5%[2])
Eastern barred bandicoot (*Perameles gunnii*)	Huon Valley, Tasmania (Obendorf et al., 1996)	July 1992 - March 1995	Direct agglutination test and modified agglutination test[9] *Both titres ≥ 1:64[1]*	10 / 150 (6.7%; 3.7 - 11.8%[2])[10,11]
Western barred bandicoot (*P. bougainville*)	Faure Island Sanctuary, Western Australia (Parameswaran, 2008)	April 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40[1]*	0 / 2 (0%; 0 - 70.8%[2])
Long-nosed bandicoot (*P. nasuta*)	Queensland (Pope et al., 1957a)	August 1955 – February 1956	Complement fixation test *Titre ≥ 1:8[7]*	1 / 1 (100%; 15.8- 98.7%[2])
Long-nosed bandicoot	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥ 1:8[1]*	2 / 7 (28.6%; 8.5 - 65.1%[2])
Greater bilby (*Macrotis lagotis*)	Queensland (Cook and Pope, 1959)	Not specified	Complement fixation test *Titre ≥ 1:8[1]*	0 / 1 (0%; 0 - 84.2%[2])
Northern quoll[6] (*Dasyurus hallucatus*)	Kakadu National Park, Northern Territory (Oakwood and Pritchard, 1999)	Feb 1993 - May 1995	Latex agglutination test	0 / 22[12] (0%; 0 - 14.8%[2])
Eastern quoll (*D. viverrinus*)	Cradoc & Judbury, Tasmania (Fancourt et al., 2014)	May 2011 - July 2013	Modified Agglutination Test (not IgM) *Titre ≥ 1:64[1]*	Ranged from 77.3% - 100%[13]

Species	Study location (study)	Sampling timeframe	Serological Test (restrictions on antibody type detected, if any) *Cut off for seropositivity*	No. seropositive / no. tested (%; 95% CI)
Eastern quoll	Bruny Island, Tasmania (Fancourt et al., 2014)	May 2011 - September 2013	Modified Agglutination Test (not IgM) *Titre ≥ 1:64*[1]	Ranged from 9.4 - 29.4%[13]
Eastern quoll	Tasmania (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:64*[1]	13[14] / 24 (54.2%; 34.9- 72.2%[2])
Spotted-tailed quolls (*D. maculatus*)	Tasmania (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:64*[1]	5[14] / 7 (71.4%; 34.9- 91.5%[2])
Western quoll / chuditch (*D. geoffroii*)	Batalling Forest, Western Australia (Haigh et al., 1994)	Not specified	Latex agglutination test	2 / 17 (11.8%; 3.6 - 34.7%[2])
Western quoll / chuditch	Julimar state forest, Western Australia (Parameswaran, 2008)	June 2007	Modified agglutination test (not IgM) *Titre ≥ 1:40*[1]	3 / 23 (13.0%; 0 - 26.8%)
Tasmanian devil (*Sarcophilus harisii*)	Tasmania[3] (Hollings et al., 2013)	Not specified	Modified agglutination test (not IgM) *Titre ≥ 1:64*[1]	6[14] / 18 (33.3%; 16.3- 56.6%[2])

[1] Cut off for seropositivity nominated by study authors.

[2] Calculated by review author using Jeffrey's 95% confidence interval.

[3] Seven red kangaroos (*Macropus rufus*) and 42 grey kangaroos *(M. giganteus)* sampled. Data as to which species the excluded anticomplementary sera were from was not reported.

[4] Anti-complementary sera excluded from results: 18 macropod sera (Cook and Pope, 1959); one common brushtail possum serum (Cook and Pope, 1959); 35 southern brown bandicoot sera (Cook and Pope, 1959); two southern brown bandicoot sera (Pope et al., 1957a).

[5] Pooled data from various locations in Tasmania.

[6] Some or all samples obtained via roadkill.

[7] Cut off applied to presented data *post hoc* by thesis author.

[8] Many possums were sampled on multiple occasions - if any individual tested positive at any time, it was included as 'positive'; all possums that tested negative on all occasions were included as 'negative'.

[9] DAT titre < 64 and no reaction on MAT classed as negative serological result; DAT ≥ 64 and no reaction on MAT classed as suspicious serological result; DAT and MAT titres both ≥ 64 classed as positive serological result.

[10] A further 7 / 150 (4.7%) had 'suspicious' reactions.

[11] One seropositive bandicoot appeared to have neurological symptoms when subsequently retrapped (unclear whether this was a blinded observation); one seropositive bandicoot found dead in trap on retrapping, and had histopathological evidence of *T. gondii* infection.

[12] Two quolls had very low serological reactions (1:2 and 1:3) that the study authors thought were probably insignificant. Neither of these quolls had histological evidence of *Toxoplasma gondii* infection.

[13] Cohort study - seroprevalence measured approximately every second month.

[14] Estimated from graph in reference.

Seroprevalence surveys are prone to limitations regarding inferring infection prevalence, similar to those affecting surveys of *T. gondii*. In particular, the potential influence of misclassification is an important consideration. While the use of serological surveys avoids invasive tissue sampling (which usually necessitates euthanasia or opportunistic sampling of otherwise dead animals in wildlife populations), it is important that the serological test(s) have been validated at the cut off titre used to differentiate infected and non-infected animals, in the species being studied. Identifying whether or not the serological test accurately reflects the infection status of the tested host is essential in estimating the true prevalence of infection from apparent seroprevalence data. Very few serological tests have been adequately validated for use in Australian marsupial species (Tables 1.7, 1.8, 1.9).

Factors that may impact the validity of using serological survey data to estimate infection prevalence include: 1) an antibody response may reflect exposure to an infection, but not necessarily establishment of infection in the host; 2) if the sampled animal is acutely infected, it may not yet have a detectable antibody titre; 3) if an infected animal has developed an IgM titre, but not yet IgG, serological tests that do not detect *T. gondii* IgM antibodies (e.g. the modified agglutination test, or some ELISAs) will not classify these animals as infected; 4) in animals with long term chronic *T. gondii* infections, serological antibody titres may drop to levels that are below the cut off titre for differentiating infected *vs.* uninfected animals, compromising sensitivity - waning serological titres have been demonstrated in chuditch (Haigh et al., 1994), brushtail possums (Eymann et al., 2006), woylies (A. Worth, Murdoch University - unpublished results) and in eastern grey kangaroos clinically suspected of *T. gondii* infection (Miller et al., 2003); 5) non-specific agglutination, which is known to occur with the direct agglutination test in other species (Dubey, 2010), may compromise test specificity; 6) anti-complementary sera require exclusion from serosurveys that use the complement fixation test, which may bias findings (e.g., Pope et al., 1957a; Cook and Pope, 1959); and 7) the complement fixation test, the Sabin Feldman dye test and ELISAs require

species-specific reagents. ELISAs used in marsupial surveys were developed for use in the species involved. However, in studies where the Sabin-Feldman dye test or complement fixation test were used, there was no description of the test being adapted for the marsupial species surveyed (Pope et al., 1957a; Cook and Pope, 1959; Gibb et al., 1966; Munday, 1972).

Table 1.7 Validation of serological tests for detecting *Toxoplasma gondii* infection in Australian marsupial species, by experimental infection

Species (study)	*T. gondii* strain (dose and route of administration; clinical outcome)	Serological test (restrictions on antibody type detected, if any)	Days post infection	Sensitivity % (95% CI[1]) *No. test +ve / no. infected*
Eastern barred bandicoots *(Perameles gunnii)* (Bettiol et al., 2000)	P89/ Veg strain (two bandicoots infected with 100 cysts orally; both died)	Direct agglutination test *Titre ≥ 1:64[2]*	6	50% (9.4 - 96.0%) *1 / 2*
			13	50% (9.4 - 96.0%) *1 / 2*
			Time of death[3]	100% (29.2- 99.2%) *2 / 2*
		Modified agglutination test (not IgM)	6	0% (0 - 70.8%) *0 / 2*
			13	0% (0 - 70.8%) *0 / 2*
			Time of death[3]	0% (0 - 70.8%) *0 / 2*
Tammar wallabies (*Macropus eugenii*) (Lynch et al., 1993)	S48 attenuated strain (two wallabies dosed with either 62 000 or 250 000 oocysts orally, two wallabies dosed with 125 000 oocysts orally; all developed severe clinical symptoms or died)[4,5]	Direct agglutination test *Titre ≥ 1:64[2]*	7	75% (28.4- 94.7%) *3 / 4*
			12	100% (47.8- 99.5%) *4 / 4*
		Modified agglutination test (not IgM) *Titre ≥ 1:40[2]*	7	0% (0 - 52.2%) *0 / 4*
			12	0% (0 - 52.2%) *0 / 4*
Eastern grey kangaroos (*M. giganteus*) (Johnson et al., 1989)	Pork I strain (two kangaroos dosed with either 50 or 500 oocysts orally, one kangaroo dosed with 250 oocysts intramuscularly[6]; all subclinically infected)[5]	Direct agglutination test *Titre > 1:32[7]*	10[8]	0% (0 - 60.2%) *0 / 3*
			20[8]	100% (39.8- 99.4%) *3 / 3*
			30[8]	100% (39.8- 99.4%) *3 / 3*

Species (study)	*T. gondii* strain (dose and route of administration; clinical outcome)	Serological test (restrictions on antibody type detected, if any)	Days post infection	Sensitivity % (95% CI[1]) *No. test +ve / no. infected*
			40[8]	100% (39.8- 99.4%) *3 / 3*
			50[8]	100% (39.8- 99.4%) *3 / 3*
		Modified agglutination test (not IgM) *Titre ≥ 1:40[2]*	10[8]	0% (0 - 60.2%) *0 / 3*
			20[8]	0% (0 - 60.2%) *0 / 3*
			30[8]	100% (19.4- 93.2%) *2 / 3*
			40[8]	100% (39.8- 99.4%) *3 / 3*
			50[8]	100% (39.8- 99.4%) *3 / 3*

[1] Calculated using Jeffrey's 95% CI.

[2] Cut off for seropositivity applied to presented data *post hoc* by thesis author.

[3] The two infected bandicoots died 15 and 17 days post infection.

[4] Data from 2 wallabies seropositive on day of inoculation excluded from sensitivity calculations.

[5] Animals included in sensitivity calculations presumed to have been successfully infected with *T. gondii*- no post mortem findings were presented confirming this to be the case.

[6] Data from one wallaby dosed with 5 oocysts orally excluded, as wallaby did not seroconvert and no post mortem evidence confirming of infection was provided.

[7] Cut off for seropositivity nominated by study authors.

[8] Time points estimated from reference graphs, which did not match reference methodological description of sampling frequency.

Table 1.8 Validation of serological tests for detecting *Toxoplasma gondii* infection in Australian marsupial species, by observational studies

Species (study)	Data origin	Serological test (Restrictions on antibody type detected, if any) *Cut off for seropositivity*	Gold standard[1]	Sensitivity % (95% CI)[2] *No. test +ves / no. gold standard +ves*	Specificity % (95% CI)[2] *No. test -ves / no. gold standard -ves*
Western grey kangaroos (*Macropus fuliginosus*) (Parameswaran, 2008)	Culled (asymptomatic) animals	ELISA (IgG only) *Optical density (OD) > 0.636*[3]	PCR (brain, and tongue or heart)	100% (69.2 - 99.7%) *9 / 9*	100% (69.2 - 99.7%) *9 / 9*
Black-faced kangaroos (*M. fuliginosus melanops)* (Dubey et al., 1988)	Naturally infected animals in captivity	Latex agglutination test *Titre ≥ 1:32*[4]	Case definition for the naturally infected animals not specified	85.7% (47.3 - 96.8%) *6 / 7*	NA
		Sabin-Feldman dye test *Titre ≥ 1:16*[4]		83.3% (42.1 - 96.3%) *5 / 6*	NA
		Indirect haem-agglutination inhibition test *Titre ≥ 1:32*[4]		85.7% (47.3 - 96.8%) *6 / 7*	NA
		Modified agglutination test (not IgM) *Titre ≥ 1:40*[4]		85.7% (47.3 - 96.8%) *6 / 7*	NA
Bennett's wallabies *(M. rufogriseus rufogriseus)* (Johnson et al., 1989)	Wild animals obtained from 5 Tasmanian locations	Direct agglutination test *Titre > 1:32*[3]	Mouse bioassay (host brain emulsion injected IP; histopathology of mouse brain, and mouse serology)	100% (59.0 - 99.6%) *6 / 6*	100% (76.8 - 99.8%) *11 / 11*
Bennett's wallabies (Johnson et al., 1988)	Wild animals obtained from 5 Tasmanian locations	ELISA (IgM & IgG)[5] *OD ≥ 0.25*[3]	Mouse bioassay (host brain emulsion injected IP; histopathology of mouse brain, and mouse serology)	100% (59.0 - 99.6%) *6 / 6*	100% (76.8 - 99.8%) *11 / 11*

Species (study)	Data origin	Serological test (Restrictions on antibody type detected, if any) *Cut off for seropositivity*	Gold standard[1]	Sensitivity % (95% CI)[2] *No. test +ves / no. gold standard +ves*	Specificity % (95% CI)[2] *No. test -ves / no. gold standard -ves*
Bennett's wallabies (Dubey and Crutchley, 2008)	Suspected naturally acquired toxoplasmosis	Modified agglutination test (not IgM) *Titre ≥ 1:40[4]*	Mouse bioassay (host brain emulsion injected SC; impression smears of mouse lung and brain, mouse serology)	100% (29.2 - 99.2%) *2 / 2*	0% (0 - 84.2%) *0 / 1[6]*
Tammar wallabies *(M. eugenii)* (Dubey and Crutchley, 2008)	Suspected naturally acquired toxoplasmosis	Modified agglutination test (not IgM) *Titre ≥ 1:40[4]*	Mouse bioassay (host brain, lung, heart and skeletal muscle emulsion injected SC; impression smears of lung and brain, and mouse serology)	100% (15.8 - 98.7%) *1 / 1*	NA
Tasmanian pademelons *(Thylogale billardierii)* (Johnson et al., 1989)	Wild animals obtained from 5 Tasmanian locations	Direct agglutination test *Titre > 1:32[3]*	Mouse bioassay (host brain emulsion injected IP; histopathology of mouse brain, and mouse serology)	100% (39.8 - 99.4%) *3 / 3*	100% (78.2 - 99.8%) *14 / 14*
Tasmanian pademelons (Johnson et al., 1989)	Suspected naturally acquired toxoplasmosis	Direct agglutination test *Titre > 1:32[3]*	Histopathology (brain)	100% (47.8 - 99.5%) *4 / 4*	NA
Tasmanian pademelons (Johnson et al., 1989)	Suspected naturally acquired toxoplasmosis	Modified agglutination test (not IgM) *Titre ≥ 1:40[4]*	Histopathology (brain)	0% (0 - 52.2%) *0 / 4*	NA

Species (study)	Data origin	Serological test (Restrictions on antibody type detected, if any) *Cut off for seropositivity*	Gold standard[1]	Sensitivity % (95% CI)[2] *No. test +ves / no. gold standard +ves*	Specificity % (95% CI)[2] *No. test -ves / no. gold standard -ves*
Tasmanian pademelons (Johnson et al., 1988)	Wild animals obtained from 5 Tasmanian locations	ELISA (IgM & IgG)[5] *OD ≥ 0.25[3]*	Mouse bioassay (host brain emulsion injected IP; histopathology of mouse brain, and mouse serology)	100% (47.8 - 99.5%) *4 / 4*	100% (76.8 - 99.8%) *13 / 13*
Tasmania pademelons (Munday, 1972)	Prevalence survey	Sabin-Feldman dye test *Titre ≥ 1:16[3]*	Mouse bioassay (host brain homogenates inoculated- details not provided)	100% (39.8 - 99.4%) *3 / 3*	NA
Common wombat (*Vombatus ursinus*) (Hartley, 2006)	Animal suffering acute toxoplasmosis	Direct agglutination test *Titre > 1:64[3]*	Histopathology (including heart, lungs, liver, kidneys) with immunohistochemistry	100% (15.8 - 98.7%) *1 / 1*	NA
		Modified agglutination test (not IgM) *Titre > 1:64[3]*		100% (15.8 - 98.7%) *1 / 1*	NA
Northern quoll (*Dasyurus hallucatus*) (Oakwood and Pritchard, 1999)	Prevalence survey (of quolls ≥ 6 months of age)	Latex agglutination test *Titre ≥ 1:32[4]*	Histopathology (brain, heart, lungs, diaphragm, liver, "gut", hind limb muscle, tongue)	NA	100% (85.2 - 100%) *22 / 22*
Crest-tailed mulgara (*Dasycercus cristicauda*) (Attwood et al., 1975)	Mix of laboratory reared and wild caught animals	Sabin-Feldman dye test *Titre > 1:16[4]*	Histopathology (brain, heart, lungs, spleen, pancreas, parapancreatic node, kidneys, adrenal gland, stomach, "gut")	100% (54.1 - 99.6%) *5 / 5*	NA
		Complement fixation test		0% (0 - 45.9%) *0 / 5*	NA
Kowari (*Dasyuroides byrnie*) (Attwood et al., 1975)	Mix of laboratory reared and wild caught animals	Sabin-Feldman dye test *Titre > 1:16[4]*	Histopathology (brain, heart, lungs, spleen, pancreas, parapancreatic node, kidneys, adrenal gland, stomach, "gut")	80% (35.9 - 95.7%) *4 / 5*	33.3% (6.8 - 80.6%) *1 / 3*
		Complement fixation test		0% (0 - 41.0%) *0 / 6*	100% (39.8 - 99.4%) *3 / 3*

Species (study)	Data origin	Serological test (Restrictions on antibody type detected, if any) *Cut off for seropositivity*	Gold standard[1]	Sensitivity % (95% CI)[2] *No. test +ves / no. gold standard +ves*	Specificity % (95% CI)[2] *No. test -ves / no. gold standard -ves*
Southern brown bandicoot (*Isoodon obesulus*) (Pope et al., 1957a)	Prevalence survey	Complement fixation test *Titre > 1:16*[4]	Mouse bioassay (suspension of post host organs, injected IP; suspension of host brain injected IC. If signs of *T. gondii* infection present in mouse at autopsy, brain impression smears examined)	87.5% (63.6 - 96.2%) *14 / 16*[7]	100% (83.2 - 99.9%) *19 / 19*[7]
Long nosed bandicoot (*Perameles nasuta*) (Pope et al., 1957a)	Prevalence survey	Complement fixation test *Titre > 1:16*[4]	Mouse bioassay (suspension of post host organs, injected IP; suspension of host brain injected IC. If signs of *T. gondii* infection present in mouse at autopsy, brain impression smears examined)	100% (15.8 - 98.7%) *1 / 1*	NA

[1] Regarding mouse bioassay inoculations: IP = intraperitoneal; SC = subcutaneous; IC = intracardiac

[2] Jeffrey's 95% CI.

[3] Cut off for seropositivity nominated by study authors.

[4] Cut off for seropositivity applied to presented data *post hoc* by thesis author.

[5] The ELISA was designed to measure both IgM and IgG. However, after considering the ELISA results in comparison to the DAT and MAT results, the authors believe that the ELISA probably only detected IgG.

[6] Wallaby negative on mouse bioassay was suspected of having clinical toxoplasmosis (supported by rising *T. gondii* serological titres) approximately two and a half years prior to time of death.

[7] Two anticomplementary sera excluded from results

Table 1.9 Data available to compare serological tests for use in detecting *Toxoplasma gondii* infection in Australian marsupial species

| Species (study) | Data origin | Serological tests compared: | | Agreement between tests |
		Test 1 (Restrictions on antibody type detected, if any) *Cut off for seropositive[1]*	Test 2 (Restrictions on antibody type detected, if any) *Cut off for seropositive[1]*	Kappa coefficient (Strength of agreement) [No. samples compared]
Western grey kangaroos (*Macropus fuliginosus*) (Parameswaran et al., 2009a)	Culled (asymptomatic) animals	ELISA (IgG only) *Optical density (OD) > 0.636*	Modified agglutination test (not IgM) *Titre ≥ 1:40*	1 (Excellent agreement) [54 samples]
Eastern grey kangaroos (*M. giganteus*) (Parameswaran, 2008)	Not described	ELISA (IgG only) *OD > 0.636*	Modified agglutination test (not IgM) *Titre ≥ 1:40*	1 (Excellent agreement) [12 samples]
Agile wallaby (*M. agilis*) (Parameswaran, 2008)	Captive wallabies (suspected toxoplasmosis outbreak)	ELISA (IgG only) *OD > 0.636*	Modified agglutination test (not IgM) *Titre ≥ 1:40*	0.91 (Excellent agreement) [45 samples]
Common wallaroo (*M. robustus*) (Riemann et al., 1974)	Captive animals (zoo)	Indirect haemagglutination test *Titre ≥ 1:64*	Complement-staining fluorescent antibody test *Titre ≥ 1:16*	Kappa coefficient not calculable (all concordant seropositives) [5 samples]
Common wombat (*Vombatus ursinus*) (Hartley and English, 2005)	Seroprevalence survey	Direct agglutination test *Titre ≥ 1:64*	Modified agglutination test (not IgM) *Titre ≥ 1:64*	1 (Excellent agreement) [23 samples]
		Direct agglutination test *Titre ≥ 1:64*	Latex agglutination test *Titre > 1:32*	0.23 (Fair agreement) [23 samples]
Eastern barred bandicoot (*Perameles gunnii*) (Miller et al., 2000)	Seroprevalence survey (captive populations)	Direct agglutination test *Titre > 1:64*	Modified agglutination test (not IgM) *Titre ≥ 1:64*	0.32 (Fair agreement) [57 samples]

[1] Cut off for seropositivity nominated by study authors, or inferred via study methodology.

1.4.2.3　　　　Prevalence data as a measure of infection frequency

Beyond the potential impact of misclassification and sample size on inferring infection prevalences from apparent prevalences / seroprevalences obtained via surveys, prevalence data are prone to substantial limitations as a measure of infection frequency, including in Australian marsupial species.

When infection is stable in a population, prevalence approximates the product of the incidence of infection, and mean duration of infection. Many surveys identified low apparent prevalences or seroprevalences. If not impacted by misclassification, these findings could reflect *T. gondii* infection being relatively uncommon in the surveyed populations. Alternatively, they may be consistent with *T. gondii* infection being relatively common but associated with high short term mortality rates in these populations, resulting in infected animals having a low probability of inclusion in prevalence surveys.

Conversely, high *T. gondii* prevalence / seroprevalence has been detected in some marsupial populations, including those subject to culls due to overpopulation. If not impacted by misclassification, such findings suggest, but do not provide conclusive evidence, that *T. gondii* infection does not substantially impact population viability. It has been postulated that other factors, particularly concurrent stressors, may interact with *T. gondii* infection to produce adverse outcomes (e.g. Obendorf and Munday, 1983; Johnson et al., 1988; Miller et al., 2000). A high prevalence of *T. gondii* infection in a stable population may therefore suggest that the population could be at risk of disastrous population impacts, if the population is subject to such factors.

1.4.3 Frequency of disease (toxoplasmosis) following infection with *Toxoplasma gondii* in free-ranging populations of Australian marsupial species

Cats were introduced into Australia from Europe in the 1800s (Denny and Dickman, 2010). It is thus assumed that the introduction and spread of *T. gondii* across Australia commenced around this time. This presumptive short history of exposure to *T. gondii* infection has been widely suggested to have resulted in increased virulence and pathogenicity of *T. gondii* in Australian marsupial species, compared to other intermediate hosts worldwide (Johnson et al., 1988; Canfield et al., 1990; Lynch et al., 1993; Reddacliff et al., 1993; Obendorf et al., 1996; Barrows, 2006; Eymann et al., 2006; Adkesson et al., 2007; Hollings et al., 2013). However, there is no evidence available to support this claim, and there is no reason to assume that the virulence of a parasite will always be greater in new host species (Ebert and Herre, 1996).

It has also been suggested that various stressors may be relevant in the clinical manifestation of *T. gondii* infections - both acute and recrudescent - in marsupials (Obendorf and Munday, 1983; Johnson et al., 1988; Miller et al., 2000; Parameswaran et al., 2010; Fancourt et al., 2014), although there are no published explorations of this hypothesis. Some reports of toxoplasmosis amongst captive marsupial populations have noted exposure to potential stressors prior to the occurrence of disease. For example, immediately prior to an outbreak associated with high mortality, a population of captive sugar gliders (*Petaurus breviceps*) was exposed to repeated episodes of social disruption, suboptimal nutrition and suboptimal temperature (Barrows, 2006). Other cases or outbreaks of toxoplasmosis in captive wallabies have occurred following relocations (Wilhelmsen and Montali, 1980; Dubey and Crutchley, 2008; Bermundez et al., 2009) or social isolation (Adkesson et al., 2007). A study of *T. gondii* infection in dasyurids (65 wild-caught, 103 laboratory reared), using histopathology without confirmatory testing, found the widest dissemination of organisms consistent with *T.*

gondii in two fat-tailed false antechinus (*Pseudantechinus macdonnellensis*) with leukaemia (Attwood and Woolley, 1973).

1.4.3.1 Longitudinal studies of the population effects of *Toxoplasma gondii* in free-ranging populations of Australian marsupials

Cohort studies are the method of choice for comparing survivability in infected *vs.* uninfected marsupial hosts, but are difficult to complete to an acceptable standard in free-ranging populations. Key challenges include: 1) the vast resources required to precisely follow up cohorts of a statistically adequate sample size; 2) the high susceptibility of wildlife cohorts to bias, particularly due to loss to follow-up, and when considering survivability, the typical requirement for a proxy for death (such as failure to retrap) which cannot differentiate the outcome from loss to follow-up; 3) intermittent surveillance of wildlife cohorts (to monitor exposure and outcome status) entails a low probability of sampling infected animals that die acutely of toxoplasmosis, and the seropositive cohort is therefore more likely to involve animals that survive with chronic infection than those which succumb to acute infection; and 4) putative confounding factors, such as age, can be very difficult to accurately measure in wild animals, and therefore accurately account for in statistical analyses, leaving study findings prone to confounding.

These challenges likely explain why only two such studies have been undertaken thus far. The first was on eastern barred bandicoots at two sites in southern Tasmania's Huon Valley. Sites were trapped every three months between July 1992 and March 1995. One hundred and fifty bandicoots were trapped over the period. Both the direct agglutination test (without 2-mercaptoethanol) (DAT) and modified agglutination test (that includes 2-mercaptoethanol) (MAT) were used. One hundred and thirty-three bandicoots were negative on both serological tests, and 68% of these were recaptured at least once. Ten bandicoots were positive on both the MAT and DAT (both titres ≥

64); five were not retrapped, while five had antibodies on two consecutive occasions (three months apart) and were not recaptured subsequently (Obendorf et al., 1996). These findings may reflect reduced survival times in eastern barred bandicoots infected with *T. gondii*, but there are substantial limitations in drawing such a conclusion. The generalities listed above apply, and lack of validation of parasite detection via the serological methodology utilised is a potential influence on findings. Additionally, the small seropositive cohort size means that chance effects on the results cannot be confidently excluded, and confounding (by age, in particular) cannot be ruled out, as the cumulative risk of *T. gondii* infection has been demonstrated to increase with age in other species (Dubey, 2010). A further seven bandicoots tested positive on the direct agglutination test (titre ≥ 64) in this study, but were negative on the modified agglutination test (titre < 64); none of these seven bandicoots were recaptured (Obendorf, et al., 1996). Again, while this may reflect a lower survival time in bandicoots acutely infected with *T. gondii*, this is not sufficient evidence to draw such a conclusion. The direct agglutination test has not been validated in eastern barred bandicoots, and it is known to have relatively poor specificity in other species (Dubey, 2010). Therefore, misclassification cannot be excluded, along with chance effects due to the small sample size.

The second study was undertaken in eastern quolls in Tasmania. Survival times of eastern quolls that were seropositive for *T. gondii* were compared to those that were seronegative, in a location where the quoll population was classified as 'stable' (Bruny Island) across a period of two years and four months. There was no evidence of a difference in survival time between seropositive and seronegative quolls (Fancourt et al., 2014). However, a lack of power cannot be excluded as an influence on study findings, due to the relatively small sample size involved. Regarding the use of these data to compare the survival time of *T. gondii* infected *vs* uninfected marsupials, the generalities described above apply, as does a lack of validation in the serological methodology used to infer host *T. gondii* infection status.

In addition to the two studies discussed above, Eymann et al. (2006) trapped brushtail possums in various locations in Sydney, on four different occasions over two and a half years. Trapped possums had their *T. gondii* serological status measured using the modified agglutination test, and were considered positive if reacting at a titre of ≥ 1:25. While this study did not aim to compare survivability of infected *vs.* uninfected hosts, and thus the use of these data for such a purpose entails many limitations, it is of interest that though 5 / 9 seropositive possums were not retrapped subsequent to testing seropositive for *T. gondii* (55.6%; Jeffrey's 95% CI 26.2 - 81.3%), the proportions of seronegative possums retrapped were similar (varying from 43 - 65% over subsequent sessions).

1.4.3.2 Using morbidity and mortality linked to toxoplasmosis in captive populations of Australian marsupials as an indicator of outcomes of *Toxoplasma gondii* infection in free-ranging populations

Relatively high morbidity and mortality rates have been reported in outbreaks of toxoplasmosis (Miller et al., 1992; Barrows, 2006; Basso et al., 2007; Dubey and Crutchley, 2008) or likely toxoplasmosis (Boorman et al., 1977; Jensen et al, 1985) in captive populations involving a number of Australian marsupial species, including sugar gliders, Bennett's wallabies, tammar wallabies (*Macropus eugenii*), common wallaroos, eastern grey kangaroos*,* red kangaroos and long-nosed potoroos (*Potorous tridactylus*). However, these outbreaks, and case studies of toxoplasmosis in captive marsupials, cannot be presumed to be representative of morbidity and mortality rates following *T. gondii* infection in free-ranging populations. Thus, they cannot be presumed to be indicative of marsupial species' inherent susceptibility to *T. gondii* infection or to toxoplasmosis, despite widespread citations of such studies to these ends in the literature (e.g., Miller et al., 1992; Reddacliff et al., 1993; Miller et al., 2003; Hartley and English, 2005; Barrows, 2006; Bermundez et al., 2009; de la Cruz-Hernandez et al., 2012).

Firstly, cases of subclinical *T. gondii* infection in captive populations are likely to go unnoticed, and hence uninvestigated and unpublished. This may lead to an inaccurate preconception that *T. gondii* infections typically manifest as clinical disease. Seropositive marsupials have been identified amongst captive populations not showing clinical signs of toxoplasmosis (Riemann et al., 1974; Jakob-Hoff and Dunsmore, 1983; Miller et al., 2000; de Camps et al., 2008) and subclinical infection consistent with *T. gondii* (not confirmed by further testing) has been demonstrated histopathologically in captive dasyurids (Attwood et al., 1975). Secondly, a substantial number of factors that may be associated with *T. gondii* morbidity and mortality may differ between captive and free-ranging populations of Australian marsupial species. These may include differences in: stress-induced immunosuppression (for example, as a result of housing circumstances, population density, nutrition and interaction with humans); average life expectancy (captive populations may include substantially older animals than would be found in the wild); the presence of co-morbidities in captive populations that may compromise an animal's immunity (and predispose the animal to clinical toxoplasmosis) but would rarely be compatible with survival in the wild; and mechanisms of exposure to *T. gondii* (particularly via dietary sources, including levels of infection and strains of *T. gondii* present in meat products that are unlikely to be common in diets of free-ranging Australian marsupials). These factors may substantially bias pertinent statistics, such as morbidity and mortality rates following *T. gondii* infection and / or the rate of recrudescence of latent infection to clinical disease.

1.4.3.3 Using morbidity and mortality in experimental *Toxoplasma gondii* infection of Australian marsupial species under laboratory conditions as an indicator of outcomes of *T. gondii* infection in free-ranging populations

Experimental studies under laboratory conditions are very useful for investigating the pathological processes of *T. gondii* infection and clinical toxoplasmosis in Australian

marsupial species, and evaluating parasite detection test accuracy. However, caution is required in extrapolating morbidity and mortality rates observed in such studies to free-ranging populations. For example, experimental infections undertaken in eastern barred bandicoots demonstrated the ability of *T. gondii* to cause fatal disease in this species (2 / 2 infected bandicoots died) (Bettiol et al., 2000). These findings may reflect eastern barred bandicoots being particularly susceptible to fatal toxoplasmosis following *T. gondii* infection. However, the widespread citation of this study as providing conclusive evidence of the high susceptibility of Australian marsupial species to toxoplasmosis is premature, for several reasons. Firstly, the study was restricted to eastern barred bandicoots, and thus assumptions that the results are applicable to all Australian marsupial species are not appropriate. Secondly, the small number of bandicoots infected is marked limitation in using the findings of this study to estimate the morbidity and mortality rates of toxoplasmosis post infection in this species. Thirdly, the inoculation dose and strain of *T. gondii* used in the experiment may not reflect the typical dose of infection, and virulence and pathogenicity, of the strains of *T. gondii* to which free-ranging populations of Australian marsupial species are commonly exposed. The bandicoots were orally infected with 100 oocysts of the P89 strain of *T. gondii,* which is highly virulent in mice (Dubey et al., 1995). The authors noted that a previous attempt to induce infection in bandicoots with a lower dose of 10 oocysts of the same strain was unsuccessful (Bettiol et al., 2000). Finally, the morbidity and mortality rates observed in this study may have been biased by circumstances related to the experiment. In particular, the bandicoots studied may have been under stress. They were captured from the wild for the purposes of the study, then housed in captivity, fed a novel diet, and subjected to handling and procedures which included an oral inoculation procedure, repeated rectal temperature monitoring and repeated blood tests. If stress-induced immunosuppression does indeed act as a causal complement in toxoplasmosis, stress may have facilitated the development of clinical disease in these bandicoots.

Studies undertaken in marsupial populations habituated to laboratory conditions prior to experimental infection may be more accurate in estimating morbidity and mortality rates associated with *T. gondii* infection in free-ranging populations, by moderating the potential impact of stress. However, findings from such studies vary. Experimental infections undertaken in four eastern grey kangaroos, as part of a validation study for *T. gondii* serological tests, did not result in clinical toxoplasmosis. Three kangaroos were infected orally with the Pork I strain of *T. gondii* (5, 50 and 500 oocysts administered, respectively) and one kangaroo was infected via intramuscular injection of 250 oocysts of the same strain. Three of the four kangaroos seroconverted during the 48 days post infection (the kangaroo dosed with 5 oocysts did not); none of the kangaroos developed clinical signs of disease within this period (Johnson et al., 1989). The interpretation of these results is limited, however, by a lack of tests to confirm the animals developed infection post inoculation (e.g. histopathology or PCR of body tissues). In contrast, in experimental infections of tammar wallabies, undertaken as part of a vaccination trial, 7 / 9 wallabies orally dosed with of 500 *T. gondii* oocysts (ME-49 strain) died 11 - 14 days post infection; 1 / 1 wallabies dosed with 1000 *T. gondii* oocysts (ME-49 strain) orally died 12 days post infection; 1 / 1 wallabies dosed with 1000 *T. gondii* oocysts (PT-12 strain) orally died 15 days post infection; and 1 / 1 wallabies dosed with 10 000 *T. gondii* oocysts (PT-12 strain) orally died 9 days post infection (Reddacliff et al., 1993). As part of another vaccination trial, six tammar wallabies were dosed with the attenuated S48 strain of *T. gondii* intramuscularly (two with 62 000, two with 125 000, and two with 250 000 tachyzoites). Ten days after inoculation, both wallabies dosed with 125 000 tachyzoites and one wallaby dosed with 250 000 tachyzoites died acutely, and one wallaby dosed with 62 000 tachyzoites was euthanized due to severe clinical illness. Post mortem findings from all animals were indicative of toxoplasmosis. The remaining two wallabies survived without clinical illness (Lynch et al., 1993).

The differences in the clinical outcome of infection between and within these studies may reflect differences in: the virulence and pathogenicity of the strains of *T. gondii* used in each study; the *T. gondii* inoculation dose (two strains of *T. gondii* have been demonstrated to have a dose-dependent pathogenicity in rats (Dubey, 1996; De Champs et al., 1998); the susceptibility of the marsupial species studied to clinical toxoplasmosis (which may or may not interact with *T. gondii* strain type or inoculation dose); or stress-induced immunosuppression of the study subjects, possibly due to inherent differences in the susceptibility of different marsupial species to stress in captivity, or to differences in management practices between the experimental settings. Further, the results of these studies cannot be confidently extrapolated to morbidity and mortality rates in free-ranging marsupial populations, due to factors such as the small numbers of animals infected, the potentially biasing influences of captivity, and inoculation doses and strains of *T. gondii* infection which may not reflect those commonly occurring in free-ranging marsupial populations.

1.4.4 Other possible effects of *Toxoplasma gondii* infection that may impact population viability of free-ranging Australian marsupials

1.4.4.1 Possible influences of *Toxoplasma gondii* on behaviour

In laboratory rats and mice, infection with *T. gondii* has been shown to increase activity level and exploratory behaviour and reduce aversion to predator odour (e.g. Berdoy et al., 2000; Vyas et al., 2007; Kannan et al., 2010). The ability to cause these behavioural changes is considered to be an adaptation of the parasite to increase transmission to the definitive host (McConkey et al., 2013; Vyas, 2013). It has been suggested that similar behavioural changes in infected marsupials may make them more vulnerable to predation by exotic predators, such as foxes and cats (Obendorf et al., 1996; Fancourt et al., 2014). Such extrapolation should be treated cautiously, however; Worth et al. (2013, 2014) document many exceptions to the commonly reported behavioural effects of *T. gondii* infection in laboratory rodents, which they

suggest are due to the use of different *T. gondii* strains, different host species and sexes, and different methodologies to measure behaviour.

Before it can be confidently asserted that infection with *T. gondii* causes behavioural changes that may increase predation risk in marsupial hosts, data from detailed behavioural studies on these hosts are required. To date, such data have not been obtained. An experimental study found infected eastern barred bandicoots were more likely to be outside their nest boxes during daylight hours, from 10 days post infection (Bettiol et al., 2000). However, the sample size was too small to confidently exclude chance effects or ensure randomisation achieved control of confounding. Additionally, measurement error and observer bias cannot be ruled out, as the method of measurement and whether observers were blinded in making these observations were not reported. Another study found *T. gondii* seroprevalence to be higher in roadkill pademelons than in culled pademelons, possibly indicating slower reaction times. However, this may have been a chance finding resulting from the small roadkill sample size (Hollings et al., 2013).

1.4.4.2 Possible effects of *Toxoplasma gondii* infection on marsupial reproductive success

From what is known of *T. gondii* infection in other species, offspring survival could be influenced by vertical transmission of the parasite. Alternatively, effects of the parasite on the reproductive fitness of mature marsupials could influence the success of breeding.

Available evidence strongly suggests that vertical transmission of *T. gondii* infection can occur in black-faced kangaroos (*M. fuliginosus melanops*), western grey kangaroos and woylies (Dubey et al., 1988; Parameswaran et al., 2009b). Vertical transmission of *T. gondii* from chronically infected western grey kangaroo dams to their young may occur commonly: two out of nine (22.2%; Jeffrey's 95% CI 6.7 - 55.6%) pouch young of chronically infected dams were positive for *T. gondii* via PCR (Parameswaran et al.,

2009b). No study has investigated the impacts of infection on the survival of vertically infected young. If vertical transmission does occur relatively frequently, and commonly results in adverse impacts on the health of infected young, reproductive success in an infected population may be compromised.

Reproductive success of mature marsupials associated with *T. gondii* infection has been investigated in one study, of eastern quolls (Fancourt et al., 2014). The mean number of pouch young in July - September was higher in females that were seropositive for *T. gondii*, than those that were seronegative. Similarly, testicular volume during the mating season was higher in seropositive males, although the implications of this finding is unclear as a relationship between testicular volume and reproductive capacity in quolls has not been demonstrated. In addition, there were difficulties in accurately measuring and controlling for putative confounding variables, which complicates interpretation of the data (Fancourt et al., 2014). Anecdotally, high prevalence of *T. gondii* was observed in populations of western grey kangaroos, red kangaroos and common wallaroos culled due to overpopulation (Parameswaran et al., 2010; Pan et al., 2012). While this suggests that *T. gondii* infection does not substantially impact reproductive capacity in these species, more specific investigations are required.

1.4.5 Future considerations for research investigating *Toxoplasma gondii* infection epidemiology in free-ranging Australian marsupial populations

T. gondii may be an important factor in population declines of marsupial species in Australia. However, this remains a hypothesis, as scientifically robust supporting evidence is not yet sufficient to draw any conclusions. Further research into this area in free-ranging populations of marsupials is required. Such research should consider:

1) Validation of diagnostic test(s) used to detect the presence or absence of *T. gondii* infection in the species under study, to enable consideration of potential impacts of

misclassification on analytical study findings (Boadella et al., 2011). Validation of tests in various marsupial species would also help to retrospectively clarify previous research findings.

2) The impact of sample sizes on results- ideally planning and undertaking studies where an adequate sample size for statistical extrapolations to a population level is realistically achievable, or where the novelty of descriptive findings would justify a study of only descriptive value. Measures of sampling error / precision should be included in results and considered in the interpretation of study findings if making analytical inferences (O'Brien et al., 2009; Boadella et al., 2011).

3) Sampling strategies, in an attempt to minimise the potential influence of selection bias (Nusser et al., 2008; Anderson, 2001; Boadella et al., 2011). Proportionate sampling is ideal, though often not realistically achievable in observational studies of wildlife.

4) Performing studies that involve temporal sequence on the same individuals (cohort studies), to compare morbidity and mortality rates of *T. gondii* infected vs non infected animals in free-ranging populations. Studies comparing findings in both stable populations and populations subject to stressors (such as habitat fragmentation) would be valuable in investigating the possibility of interaction between stressors and the impact of *T. gondii* infection on population health.

5) Controlling for confounding variables when making statistical extrapolations (for example, controlling for sex and age if using a Poisson regression to compare survivability of *T. gondii* infected and uninfected hosts). In most marsupial species, further research aimed at identifying and validating measures of potential confounders - particularly age - is required.

6) Potential influences of data clustering, which is potentially common in wildlife studies but rarely considered when making analytical inferences.

1.5 Alimentary tract parasites and macroscopic ectoparasites known to infect quenda / southern brown bandicoots (*Isoodon obesulus*)

A range of parasites of the alimentary tract, and macroscopic ectoparasites, have been documented infecting quenda / southern brown bandicoots in Australia. These are summarised in Tables 1.10 and 1.11. Investigations of parasites infecting quenda in Western Australia are limited, as are knowledge of the lifecycles of these native parasites, their pathological effects in quenda, and their epidemiology within quenda populations.

Table 1.10 Alimentary tract parasites of quenda / southern brown bandicoots (*Isoodon obesulus*)

Parasite	Reference	Documented in quenda in Western Australia	Hosts additional to native wildlife
Gastrointestinal protozoan parasites (subkingdom Protozoa):			
Entamoeba sp.	Adams, 2003	Yes	?[1]
Giardia duodenalis	Thompson et al., 2010a	Yes	Wide range of mammals, incl. humans
Giardia canis (genotype C)	Thompson et al., 2010a	Yes	Dogs
Giardia bovis	Thompson et al., 2010a	Yes	Ungulate livestock
'Quenda genotype' of *Giardia*	Adams et al., 2004; Thompson et al., 2010a	Yes	-
Cryptosporidium sp.	Dowle et al., 2013	No	?[1]
Eimeria quenda	Bennett & Hobbs, 2011	Yes	-
Novel *Eimeria* spp.	Bennett & Hobbs, 2011	Yes	?[1]
Alimentary tract nemathelminthes[2] (Phylum Nemathelminthes):			
Beveridgiella iota	Humphery-Smith, 1980	No	-
Austrostrongylus sp.	Mawson, 1960	No	-
Copemania darwini	Beveridge and Durette-Desset, 2009	Yes	-
Asymmetracantha tasmaniensis	Mawson, 1960	No	-
Mackerrastrongylus mawsonae	Inglis, 1968	Yes	-
Mackerrastrongylus peramelis	Mawson, 1960	No	-
Tetrabothriostrongylus mackerrasae	Jones et al., 1991	No	-
Peramelistrongylus skedastos	Mawson, 1960	No	-
Linstowinema cinctum	Smales, 1997; Inglis, 1968	Yes	-
Linstowinema inglisi	Smales, 1997	Yes	-

Parasite	Reference	Documented in quenda in Western Australia	Hosts additional to native wildlife
Linstowinema tasmaniense	Smales, 1997	No	-
Linstowinema warringtoni	Smales, 1997	No	-
Labiobulura baylisi	Mawson, 1960	No	-
Labiobulura inglisi	Mawson, 1960; Inglis, 1968	Yes	-
Physaloptera sp.	Spratt, 1987; Norman & Beveridge, 1999	No	?[1]
Parastrongyloides australis	Mawson, 1960	No	-
Trichuris peramelis	Mawson, 1960	No	-
Capillaria sp.	Mawson, 1960	No	?[1]
Gastrointestinal Platyhelminthes[2] (Phylum Platyhelminthes):			
Potorolepis isoodontis	Vaucher et al., 1984; Spassky, 1994;	Yes	-
Potorolepis peramelidarum	Vaucher et al., 1984; Spassky, 1994	No	-
Brachylaima simile	Cribb, 1992	No	-
Gastrointestinal acanthocephalans (Phylum Acanthocephala):			
Australiformis semoni	Schmidt and Edmonds, 1989	No	-

[1] Dependent on species, which was not determined.

[2] Table only includes helminths where quenda are the definitive host.

Table 1.11 Macroscopic ectoparasites of quenda / southern brown bandicoots (*Isoodon obesulus*)

Parasite	Reference	Documented on quenda in Western Australia	Hosts additional to native wildlife
Fleas (Order Siphonaptera):			
Acedestia chera	Dunnet & Mardon, 1974	Yes	-
Pygiopsylla hoplia	Dunnet & Mardon, 1974; Weaver & Aberton, 2004	No	Cattle, rabbits, introduced rats
Pygiopsylla tunneyi	Dunnet & Mardon, 1974	Yes	-
Pygiopsylla zethi	Dunnet & Mardon, 1974	No	-
Bibikovana iridis	Dunnet & Mardon, 1974	No	Introduced rats
Bibikovana rainbowi	Dunnet & Mardon, 1974; Weaver & Aberton, 2004	No	Introduced rats
Bibikovana sinuata	Dunnet & Mardon, 1974	No	-
Ctenocephalides felis	Dunnet & Mardon, 1974	No	Wide range, including dogs, cats and humans
Choristopsylla ochi	Dunnet & Mardon, 1974	No	Introduced rats
Stephanocircus dasyuri	Dunnet & Mardon, 1974; Weaver & Aberton, 2004	No	Introduced rats, cats
Stephanocircus harrisoni	Dunnet & Mardon, 1974;	No	-
Acanthopsylla rothschildi	Weaver & Aberton, 2004	No	-
Macropsylla hercules	Weaver & Aberton, 2004	No	Introduced rats
Lice (Order Phthiraptera):			
Boopia bettongia	von Kéler, 1970	Yes	-
Ticks (Suborder Ixodoidea):			
Ixodes tasmani	Roberts, 1970; Weaver & Aberton, 2004	No	Introduced rats, domestic animals, humans
Ixodes holocyclus	Roberts, 1970; Dowle, 2012	No	All domestic animals, poultry, humans, rodents
Ixodes fecialis	Roberts, 1970	Yes	Introduced rats and mice
Ixodes trichosuri	Dowle, 2012	No	-
Haemaphysalis bancrofti	Dowle, 2012	No	cattle, pigs, humans
Haemaphysalis sp.[1]	Weaver & Aberton, 2004	No	?
Mesostigmatan mites (Order Mesostigmata):			
Gymnolaelaps annectans	Domrow, 1987	No	Introduced rats
Haemolaelaps flagellatus	Domrow, 1987	No	-
Haemolaelaps marsupialis	Domrow, 1987	Yes	Rabbits
Mesolaelaps antipodianus	Domrow, 1987	No	Rabbits
Mesolaelaps anomalus	Weaver & Aberton, 2004	No	-
Mesolaelaps australiensis	Dowle, 2012	No	Introduced rats and mice
Mesolaelaps sminthopsis	Weaver & Aberton, 2004	No	-
Myonyssus decumani	Domrow, 1987	No	-
Androlaelaps marsupialis	Weaver & Aberton, 2004	No	-
Prostigmatan mites (Suborder Prostigmata):			
Neotrombicula novaehollandiae	Domrow & Lester, 1985	No	Dogs

[1]*Haemaphysalis humerosa* or *H. ratti* (Weaver and Aberton, 2004)

1.6 Alimentary tract parasites and macroscopic ectoparasites known to infect brushtail possums (*Trichosurus vulpecula*)

A range of parasites of the alimentary tract, and macroscopic ectoparasites, have been documented infecting brushtail possums. These are summarised in Tables 1.12 and 1.13. However, as for quenda, investigations of parasites of brushtail possums in Western Australia are limited, and no studies have investigated parasites of brushtail possums in Perth. Knowledge of the lifecycles of the native brushtail possum parasites, the pathological effects of those parasites on brushtail possums, and the epidemiology of parasites in brushtail possum populations, are also limited.

Table 1.12 Alimentary tract parasites of brushtail possums (*Trichosurus vulpecula*)

Parasite	Reference	Documented in brushtail possums in Western Australia	Hosts additional to native wildlife
Gastrointestinal protozoan parasites (Subkingdom Protozoa):			
Entamoeba spp.	Adams, 2003; Clarke, 2011	Yes	?[1]
Giardia duodenalis	Thompson (J) et al., 2008	No	Wide range of mammals, incl. humans
Cryptosporidium spp.	Hill et al., 2008a	No	?[1]
Eimeria trichosuri	O'Callaghan & O'Donoghue, 2001; Power et al., 2009	No	-
Alimentary tract nemathelminthes[2] (Phylum Nemathelminthes):			
Paraustrostrongylus trichosuri	Mawson, 1973	No	-
Trichostrongylus colubriformis	Bearup & Bolligel, 1949; O'Callaghan & Moore, 1986	No	Ruminants
Trichostrongylus axei	O'Callaghan & Moore, 1986	No	Ruminants, other mammals, birds
Trichostrongylus rugatus	Bearup & Bolligel, 1949;	No	Ruminants
Trichostrongylus retortaeformis	Beveridge & Spratt, 1996	No	Ruminants
Trichostrongylus vitrines	Gordon & Sommerville, 1958	No	Ruminants
Cooperia curticei	Gordon & Sommerville, 1958	No	Ruminants
Nematodirus sp.	Gordon & Sommerville, 1958	No	Ruminants
Filarinema trichosuri	Johnston and Mawson, 1939	No	-
Peramelistrongylus sp.	Viggers & Spratt, 1995	No	-
Profilarinema hemsleyi	Durette-Desset & Beveridge, 1981	Yes	-
Parastrongyloides trichosuri	Mackerras, 1959	No[3]	-
Strongyloides sp.	Gordon & Somerville, 1958	No	-

Parasite	Reference	Documented in brushtail possums in Western Australia	Hosts additional to native wildlife
Adelonema trichosuri	O'Callaghan & Moore, 1986	No	-
Gongylonema sp.	Viggers & Spratt, 1995	No	-
Protospirura marsupialis	Johnston and Mawson, 1939	No[3]	-
Gastrointestinal Platyhelminthes[2] (Phylum Platyhelminthes):			
Bertiella trichosuri	O'Callaghan & Moore, 1986	No[3]	-

[1] Dependent on species involved, which was not determined.

[2] Table only includes helminths where brushtail possums are the definitive host.

[3] Clarke (2011) identified eggs of the respective genus in faecal samples from brushtail possums in Western Australia.

Table 1.13 Macroscopic ectoparasites of brushtail possums (*Trichosurus vulpecula*)

Parasite	Reference	Documented on brushtail possums in Western Australia	Hosts additional to native wildlife
Fleas (Order Siphonaptera):			
Pygiopsylla hoplia	Dunnet & Mardon, 1974	No	Cattle, rabbits, introduced rats
Choristopsylla ochi	Clarke, 2011	Yes	Introduced rats
Ctenocephalides felis felis	Dunnet & Mardon, 1974; Webster et al., 2014	No	Wide range, incl. dogs, cats and humans
Acanthopsylla pavida	Dunnet & Mardon, 1974	No	-
Acanthopsylla rothschildi rothschildi	Dunnet & Mardon, 1974	No	-
Pulex irritans	Dunnet & Mardon, 1974	No	Wide range, incl. humans, dogs, pigs, fowl.
Echidnophaga myrmecobii	Clarke, 2011	Yes	Wide range of mammals incl. humans
Echidnophaga gallinacea	Dunnet & Mardon, 1974	No	Wide range of birds and mammals, incl. humans
Ticks (Suborder Ixodoidea):			
Ixodes tasmani	Roberts, 1970; Webster et al., 2014	No	Introduced rats, domestic animals, humans
Ixodes holocyclus	Roberts, 1970; Webster et al., 2014	No	Domestic animals, poultry, humans, rabbits, introduced rats
Ixodes trichosuri	Roberts, 1970; Webster et al., 2014	No	-
Haemaphysalis bremneri	Roberts, 1970	No	Cattle, horses

Parasite	Reference	Documented on brushtail possums in Western Australia	Hosts additional to native wildlife
Haemaphysalis bancrofti	Roberts, 1970	No	Cattle, sheep, pigs, dogs, humans, horses
Haemaphysalis humerosa[2]	Roberts, 1970	No	Introduced rats, humans
Amblyomma triguttatum	Clarke, 2011	Yes	Introduced rats, rabbits, domestic mammals, humans
Mesostigmatan mites (Order Mesostigmata):			
Haemolaelaps sisyphus	Domrow, 1987	No	-
Laelapsella humi	Domrow, 1987	No	Introduced rats
Trichosurolaelaps crassipes	Domrow, 1987; Webster et al., 2014	No[3]	-
Ornithonyssus sp.	O'Callaghan & Moore, 1986	No	?[1]
Prostigmatan mites (Suborder Prostigmata)			
Asoschoengastia rattus	Domrow & Lester, 1985	No	Introduced rats
Eutrombicula hirsti	Domrow & Lester, 1985	No	Humans
Neotrombicula novaehollandiae	Domrow & Lester, 1985	No	Dogs
Guntheria peregrine	Domrow & Lester, 1985	Yes	-
Guntheria trichosuri	Domrow & Lester, 1985	No	-
Guntheria pseudomys	Domrow & Lester, 1985	No	-
Guntheria shieldsi	Domrow & Lester, 1985	No	Introduced rats
Guntheria kallipygos	Domrow & Lester, 1985	No	Introduced rats
Trombicula quadriensis	Domrow & Lester, 1985	No	-
Odontocarus sp.	Domrow, 1991	No	?[1]

[1] Dependent on species involved.

[2] Record of *H. humerosa* on brushtail possums is of some doubt, due to possible confusion with *H. bremneri* (Roberts, 1970).

[3] *Trichosurolaelaps* sp. has been identified from brushtail possums in Western Australia (Clarke, 2011).

1.7   Aims and objectives

This project aimed to:

1) Investigate the impacts of urbanisation on *Toxoplasma gondii*, gastrointestinal parasite and macroscopic ectoparasitic infections (particularly anthropozoonoses, with a focus on *Giardia* spp. and *Toxoplasma gondii*) in quenda and brushtail possums in the greater Perth region, Western Australia.

2) Investigate the impact of urbanisation on morphometrics and reproduction in quenda and brushtail possums in the greater Perth region.

The objectives were to:

1) Validate the use of the modified agglutination test in detecting *Toxoplasma gondii* infection in quenda and brushtail possums.

2) Validate the use of immunofluorescence microscopy and a PCR and sequencing protocol in detecting *Giardia* spp. infection in quenda.

3) Validate the use of a faecal flotation protocol in detecting gastrointestinal helminths present in quenda.

4) Identify and estimate the prevalence of *Giardia* spp., other gastrointestinal protozoans, gastrointestinal helminths and macroscopic ectoparasites, and the seroprevalence of *T. gondii*, in quenda and brushtail possums in the greater Perth region.

5) Compare the prevalence, odds of infection and intensity of parasitic infections, and parasite communities, between quenda trapped in urbanised environments *vs.* bushland in Perth.

6) Compare the prevalence of parasitic infections, and the odds of endo- and ecto-parasitism, between brushtail possums trapped in urbanised environments *vs.* bushland in Perth.

7) Compare pouch activity between adult females trapped in urbanised environments *vs.* bushland in Perth, in quenda and brushtail possums (respectively).

8) Compare morphometrics between quenda trapped in urbanised environments *vs.* bushland in Perth.

9) Identify the types of interactions that occur between humans and urban dwelling marsupials on Perth private properties, that may play a role in transmission of infections between humans and urban dwelling marsupials.

10) Investigate the role of pet cats as a potential source of *T. gondii* infection to urban dwelling marsupials in the greater Perth region, by estimating the seroprevalence of *T. gondii*, identifying risk factors for *T. gondii* infection, and investigate proportions of pet cats managed in ways relevant to public health.

1.8 References

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Chapter 2

Seroprevalence of *Toxoplasma gondii* in pet cats in Perth, Western Australia, and cat management practices relevant to public health

2. Seroprevalence of *Toxoplasma gondii* in pet cats in Perth, Western Australia, and cat management practices relevant to public health

2.1 Introduction

Perth, Western Australia, is a city with diverse urban and semi-urban habitats, where humans, cats, other domestic animals and wildlife live in close proximity. The management of pet cats therefore potentially impacts the health of all parties, where management practices facilitate the transmission of infections from cats. For example, allowing cats outside without restrictions facilitates contamination of the environment with cat faeces, which may be a source of a variety of pathogens, including *Toxoplasma gondii.*

T. gondii is an ubiquitous protozoan parasite of concern to human and animal health. Cats are the definitive host, and many warm blooded animals are susceptible to infection as intermediate hosts. *T. gondii* infection can be transmitted via ingestion of oocysts originating from infected cat faeces, ingestion of *T. gondii* bradyzoites in infected raw or undercooked infected meat, and vertical transmission from infected mother to foetus / suckling young (Dubey, 2010). The only published data regarding *T. gondii* infection in Perth domestic cats originates from abandoned or stray cats (Jakob-Hoff and Dunsmore, 1983; Shaw et al., 1983).

In Perth pet cats, we aimed to investigate:

1) the proportion of pet cats managed in ways relevant to veterinary public health;
2) the seroprevalence of *T. gondii*; and
3) risk factors for exposure to *T. gondii*.

2.2   Methods

The target population was pet cats residing in the Statistical Division of Perth. The study population was sourced from pet cats attending Perth veterinary clinics. All (n = 133) veterinary general practices within Perth were invited to participate in the survey via mail; five clinics collected samples between February and July 2013. Cat owners attending the clinics during the study period were invited to participate in the survey. Samples submitted from stray cats and warehouse cats were excluded.

Cat owners were asked to complete a questionnaire regarding the cat's demographic information, health, healthcare, diet and living circumstances. Directly prior to questions regarding the feeding of raw meat, it was specified that tinned pet food should not be considered raw meat. A socioeconomic status proxy was created using the owner's residential address, by means of the Australian Bureau of Statistics' Index of Relative Socio-Economic Advantage and Disadvantage at a Statistical Local Area level (ABS, 2015).

One mL of blood was collected from participating cats. Serum was separated out by centrifugation, and frozen at -20°C until analysis. Serum samples were analysed for *T. gondii* antibodies using the modified agglutination test (bioMérieux, France), according to manufacturers' directions. A sample was considered seropositive if it was positive at titres of 1:40 and / or 1:4000.

Proportions of cats subject to different management practices and *T. gondii* seroprevalence were calculated with Jeffrey's 95% confidence interval. For risk factor analyses, seropositive cats were considered to have been "exposed" to *T. gondii;* seronegative cats were considered "not exposed"*,* and serum samples that had 'borderline' reactions were defaulted to "not exposed". For age, cats were grouped into those less than 1.5 years old, and those 1.5 years or older. Univariable logistic regression was undertaken using Stata 14.0 (StataCorp, 2015), with Wald p-values.

Cat sampling was undertaken under Murdoch University Animal Ethics Permit R2530/12; surveys were undertaken under Murdoch University Human Research Ethics Permit 2012/180.

2.3 Results

2.3.1 Surveyed cats - descriptive findings

Questionnaires were received for 49 cats from 47 households. The primary reasons for these cat attending the veterinary clinic when surveyed were: vaccination (14 cats); blood test (10 cats); neutering (7 cats); health check (6 cats); injury (4 cats); dental treatment (2 cats); or arthritis, biopsy, eye mass, urinary tract infection, chronic skin condition or boarding (1 cat each). Nine sampled cats were one year or younger, 23 cats were between one and 10 years old, 15 cats were 10 years or older, and age was not provided for two cats. Sixteen sampled cats were female and 33 were male. All cats were either neutered or due to be neutered at the time of surveying. Eleven cats had been adopted - nine were adopted at least two years prior to the survey. Three cats were reported as having an illness which required medication and / or regular blood tests (epilepsy, diabetes, and undescribed illness, respectively).

2.3.2 The proportion of pet cats managed in ways relevant to veterinary public health

The proportions of surveyed pet cats managed in ways relevant to veterinary public health are listed in Table 2.1. Substantial proportions of cats had not been wormed in the previous 12 months, were allowed to roam (go outside without restrictions), scavenged or predated wildlife, did not use litter trays, and were fed raw meat.

Table 2.1 The proportion of Perth pet cats managed in ways relevant to veterinary public health

Management practice		No. cats / total (%)	95% CI
Number of times cat wormed in the previous year[1]	0	12 / 47 (25.5%)	15.3 - 39.6%
	1-3	20 / 47 (42.6%)	29.5 - 56.8%
	4+	15 / 47 (31.9%)	20.4 - 46.3%
Of cats allowed to roam[2]: number of times cat wormed in the previous year[3]	0	6 / 27 (22.2%)	10.7 - 41.0%
	1-3	16 / 27 (59.3%)	40.6 - 75.5%
	4+	5 / 27 (18.5%)	8.3 - 36.9%
Cat lives with another cat(s)	No	34 / 49 (69.4%)	55.4 - 80.5%
	Yes	15 / 49 (30.6%)	19.5 - 44.6%
Cat allowed to roam[2]	No	21 / 49 (42.9%)	30.0 - 56.8%
	Yes	28 / 49 (57.1%)	66.3 - 88.5%
Cat known to predate or scavenge wildlife[4]	No	26 / 49 (53.1%)	39.3 - 66.3%
	Yes	23 / 49 (46.9%)	33.7 - 60.7%
Cat uses a litter tray	No	23 / 49 (46.9%)	33.7 - 60.7%
	Yes	26 / 49 (53.1%)	39.3 - 66.3%
Cat known to drink out of sources other than water bowl	No	28 / 49 (57.1%)	43.2 - 70.0%
	Yes	21 / 49 (42.9%)	30.0 - 56.8%
Cat fed raw kangaroo meat	No	42 / 49 (85.7%)	73.3 - 92.8%
	Yes	7 / 49 (14.3%)	7.2 - 26.7%
Cat fed raw chicken meat	No	34 / 49 (69.4%)	55.4 - 80.5%
	Yes	15 / 49 (30.6%)	19.5 - 44.6%
Cat fed raw bones	No	46 / 49 (93.9%)	83.5 - 97.8%
	Yes	3 / 49 (6.1%)	2.2 - 16.5%
Cat fed raw beef	No	40 / 49 (81.6%)	68.6 - 90.0%
	Yes	9 / 49 (18.4%)	10.0 - 31.4%
Cat fed raw lamb/mutton	No	45 / 49 (91.8%)	80.8 - 96.7%
	Yes	4 / 49 (8.2%)	3.3 - 19.2%
Cat fed raw pork	No	47 / 49 (95.9%)	86.3 - 98.7%
	Yes	2 / 49 (4.1%)	1.3 - 13.7%
Cat fed any raw red or white meat products[5]	No	28 / 49 (57.1%)	43.2 - 70.0%
	Yes	21 / 49 (42.9%)	30.0 - 56.8%
Cat fed raw fish	No	41 / 49 (83.7%)	70.9 - 91.4%
	Yes	8 / 49 (16.3%)	8.6 - 29.1%

[1] Data missing from two cats.

[2] Cat allowed to roam = cat allowed outside without restrictions: cats confined to cat enclosures, enclosed verandahs or balconies included under 'no'.

[3] Worming history missing from one cat.

[4] Cat known to predate or scavenge any/all of mice, rats, birds, reptiles or other mammals.

[5] Including raw bones and offal.

2.3.3   *Toxoplasma gondii* seroprevalence

Serum samples were obtained from 51 cats. Eighteen cats were seropositive (35.3%; 95% CI 23.6 - 49.1) and a further three cats were borderline (5.9%; 95% CI 2.1 - 15.9).

2.3.4 Risk factors for exposure to *Toxoplasma gondii*

Two cats were excluded from the risk factor analyses due to missing questionnaires. One excluded cat was *T. gondii* seronegative, and the other was seropositive.

No kittens (≤ 1 year old) were seropositive for *T. gondii*. Univariable risk factor analysis results, where there was at least weak evidence of an association with exposure to *T. gondii* (p ≤ 0.10), are listed in Table 2.2. Findings included differing odds of exposure to *T. gondii* based on age, sex and whether the cat was fed raw meat or not. This study provided no evidence against the null hypothesis (p > 0.10) regarding other factors assessed (results not shown).

Table 2.2 Univariable risk factor analyses regarding exposure to *Toxoplasma gondii* in Perth pet cats

Risk factor		Crude OR	95% CI	p-value
Sex	Male	1		
	Female	4.02	1.13 – 14.3	0.032
Age	< 1.5 years old	1		
	≥ 1.5 years old	7.14	0.82 – 61.9	0.074
Number of times cat wormed in past year	None	1		
	Once or more	8.25	0.96 – 71.1	0.055
Cat fed any raw meat products[1]	No	1		
	Yes	4.03	1.16 – 14.0	0.028
Cat fed raw kangaroo meat	No	1		
	Yes	6.25	1.06 – 36.7	0.043
Cat fed raw chicken meat	No	1		
	Yes	3.17	0.89 – 11.3	0.074

[1] Including red meat, white meat, raw bones and offal; excluding raw fish.

2.4 Discussion

This study presents the first available data regarding veterinary public health and the epidemiology of *T. gondii* infection in Perth pet cats.

2.4.1 The proportion of pet cats managed in ways relevant to veterinary public health

Although the sample size was small and our estimates of the frequency of management practices consequently lacked precision, our study suggests that in Perth, pet cat management practices that represent possible veterinary public health risk may be common. For example, pet cats commonly being allowed to roam outdoors without restrictions and not using a litter tray facilitates contamination of the environment with faecal-borne pathogens. Approximately half the pet cats in this study were known to predate or scavenge wildlife (this is likely to be an underestimate, given the probability that some owners do not see evidence of their cats' predation / scavenging activities). Similarly, a substantial proportion of cats were fed raw meat or fish products. These practices may result in the cats acting as an intermediary in transferring feral animal and wildlife-based infections, or various raw meat or fish borne infections, to humans. The proportion of cats reportedly wormed at least once in the past year is almost identical to that reported in a previous telephone survey of Perth pet cat owners (Robertson et al., 1991). Less than a fifth of cats who were allowed to roam outdoors without restrictions had a recent worming history of once every three months, without even considering the efficacy of the worming product used. As the cat owners included in this survey are likely to represent the more conscientious and educated cat owners in Perth regarding pet health, having been recruited through veterinary practices, this may be an overestimate of worming frequency of Perth pet cats in general.

2.4.2 *Toxoplasma gondii* seroprevalence

Findings suggest that Perth pet cats are commonly exposed to *T. gondii* infection, as the modified agglutination test is an accurate indicator of prior *T. gondii* infection in cats (Dubey et al., 1995; Macri et al., 2009; Györke et al., 2011). We cannot rule out bias due to the use of non-proportionate sampling, and particularly as a result of sourcing pet cats via veterinary clinics. However, it is of note that seroprevalence in abandoned

or stray Perth cats sampled from refuges was very similar in a previous study (Jakob-Hoff and Dunsmore, 1983).

2.4.3   Risk factors for exposure to *Toxoplasma gondii*

These data are consistent with potentially substantially increased risk associated with being fed raw meat - in particular, chicken and kangaroo meat. The lack of precision in the measures of effect, and the relatively high p-values, may reflect a lack of power due to the small sample size. Both these meats are known sources of *T. gondii* in Western Australia (Parameswaran et al, 2010; Chumpolbanchorn et al., 2013). A previous survey estimated that 20% of Perth pet cats are fed chicken and 12.5% are fed kangaroo, with half of cats fed meat being fed it raw (Robertson, 1999).

Further investigation into risk factors for *T. gondii* infection in Perth pet cats is required, with more cats sampled from a range of sources. Confounding could not be considered in a useful manner by multivariable analyses, due to the small survey sample size resulting in a lack of power. Confounding may have influenced associations where univariable analysis provided evidence against the statistical null hypothesis - we believe this is highly likely to be the case regarding the increased risk of *T. gondii* exposure in cats wormed at least once in the preceding year.

2.5   Conclusions

The management of pet cats may be contributing a substantial veterinary public health risk in Perth.

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Chapter 3

Interactions between humans and urban-adapted marsupials on private properties in the greater Perth region

This chapter is a published short communication:

Hillman, A., Thompson, R.C.A., 2016. Interactions between humans and urban-adapted marsupials on private properties in the greater Perth region. *Australian Mammalogy*, 38, 253-255.

Author contributions:

Designed the study: AH, RCAT

Organised and undertook data collection: AH

Analysed the data: AH

Wrote and revised the manuscript: AH

Proofed and critically appraised the manuscript: RCAT

3. Interactions between humans and urban-adapted marsupials on private properties in the greater Perth region.

3.1   Introduction

In Perth, the marsupials quenda (or southern brown bandicoots, *Isoodon obesulus)* and brushtail possums (*Trichosurus vulpecula*) remain in many areas despite urbanisation. Human interaction with such wildlife is specifically discouraged by the Western Australian government agency responsible for wildlife (Land for Wildlife / Department of Conservation and Land Management, 2005; Department of Parks and Wildlife, 2013). This is due to concerns about potential adverse impacts of interactions on wildlife health. Direct adverse impacts of supplemental feeding on wildlife may include nutritional imbalances, leading to metabolic disorders and / or obesity, and juvenile animals becoming dependent on anthropogenic food sources. Indirectly, supplemental food availability may lead to an abnormally increased marsupial population density, which may result in problems such as increased aggression and increased infection transmission. Further, wildlife losing their fear of humans and pets may increase their vulnerability to abuse and predation. Additional concerns around human-wildlife interactions are transfer of infections between humans, domestic pets and wildlife, which may have adverse consequences for all parties (Orams, 2002; Bradley and Altizer, 2007).

Despite these recommendations, interactions between these marsupials and humans are known to occur. For example, in south west Western Australia, quenda are sometimes offered food by members of the public, and have access to other anthropogenic food sources (Howard et al., 2014). Documenting the range of interactions that occurs between humans and these marsupials in Perth would help to guide public education messages designed to protect the health of marsupials, domestic pets and humans cohabiting in urban areas.

We aimed to document a range of interactions that people in Perth have with quenda and brushtail possums on their property. This included documenting the types of food offered to or scavenged by quenda and possums, and documenting other types of interactions that occur between humans and these marsupials.

3.2   Methods

All private properties participating in a Perth quenda and brushtail possum parasite survey from March 2013 to December 2014 were invited to participate in this wildlife interactions survey. Properties were recruited to the parasite survey via non-proportionate methods - primarily via a register held by WWF-Australia from the Community Quenda Survey 2012, run in conjunction with the Department of Parks and Wildlife.

One questionnaire was completed per property, by the property residents, or by the property owners in the case of private non-residential properties. The questionnaire asked both closed and open questions. Closed questions inquired about: the presence of quenda, brushtail possums, dogs, cats and chickens on the property; whether the property owners offered quenda and / or possums food; whether quenda or possums ate food intended for the dogs, cats or chickens; and whether people living on the property had any other interactions with the marsupials. Open questions asked respondent to describe: the types of food they offered to the marsupials; the types of dog / cat / chicken food that the marsupials scavenged; and any other types of interactions that people had with these marsupials on the property. Questions asked respondents to specify whether any listed meat products were raw or cooked.

This survey was undertaken under Murdoch University Human Research Ethics Committee permit 2013/167.

3.3 Results

Of the 29 private properties involved in the parasite survey, questionnaires for this wildlife interactions survey were received from owners or residents of 27 properties (26 residential and one non-residential). Questionnaires were not received from two residential properties. Twenty six respondents reported having quenda on their property, and 14 respondents reported having brushtail possums (13 respondents reported having both quenda and possums).

3.3.1 Foodstuffs specifically offered to marsupials by property residents

Twelve respondents (44%) reported specifically offering quenda and / or possums food. Foodstuffs offered included vegetables, fresh and dried fruits, bread, rice, muesli, wholegrains, rolled oats, horse muesli, seeds, nuts, cheese, cooked meat bones, cooked meat scraps, cooked meat fat and cooked leftover table scraps.

3.3.2 Anthropogenic foodstuffs taken from properties by marsupials

Nine respondents kept chickens on their property; all reported having quenda on their property, and six reported having possums. Seven respondents (78%) reported quenda and / or possums eating food intended for the chickens. Such foodstuffs included commercial poultry foods, cracked corn, wheat, fruit and vegetable scraps, porridge, bread, noodles, cooked meat (sausage, steak, chicken skin), raw bacon rind and other dinner scraps.

Eight respondents owned cats; all reported having quenda on their property, and four reported having possums. Three of the eight properties (38%) reported quenda and / or possums scavenging cat food, which was described as commercial cat biscuits. Seven respondents owned dogs; all reported having quenda on their property, and four

reported having possums. None reported quenda and / or possums scavenging the dog food.

Though no specific questions addressed backyard pigs or aviary birds, one respondent volunteered that quenda entered the backyard pig's pen to eat the goat muesli fed to the pig, and that quenda scavenged bird seed thrown out of cages by a pet cockatoo and galah. Another respondent reported quenda scavenging from an open compost heap on their property. One respondent described quenda regularly scratching around an area of the backyard that is specifically used for the disposal of old cooking oil. Another respondent reported quenda on the property "feasting on Multiguard iron pellets" (presumably the iron EDTA version of commercial snail and slug pellets).

3.3.3   Interactions between quenda and residents on private properties

Of respondents who reported quenda on their property, ten (38%) described interacting with the quenda in ways additional to offering food.

Three respondents (12%) reported that quenda entered the house when they got the chance. In all cases, it was specified that the quenda were not encouraged to do so. In the house, quenda scavenged food off the floor, rummaged through the rubbish or "ripped open packets of food on the teenager's floor". One respondent described having to chase / order the quenda out.

Four respondents (15%) reported that quenda would approach humans. One respondent reported that quenda were skittish in doing so. In two cases, it was specified that this particularly occurred when residents were eating. One respondent would occasionally feed quenda on such occasions, while the other respondent stated that the quenda scavenged scraps on the ground.

Two respondents (8%) reported occasionally hand feeding quenda, and three (12%) described occasionally patting quenda (one when hand feeding). One of the respondents who hand fed quenda stated that some quenda would come when called.

One respondent described removing ticks from the quenda, if the quenda would allow it. Another respondent reported accidentally trapping a quenda when trying to trap the neighbours' cat.

3.3.4 Interactions between brushtail possums and residents on private properties

Of respondents who reported having brushtail possums on their property, two (14%) reported interacting with possums in ways additional to offering food. One respondent described having to remove, by hand, a possum that had entered the house. Another respondent reported that possums on the property would come when called, and were sometimes hand fed. Several of these possums were described as being comfortable to sit on the residents' laps or shoulders when being fed.

3.4 Discussion

The findings of this survey - that a substantial proportion of respondents offer quenda and/or possums food - concur with the findings of a larger telephone survey of members of the public regarding quenda in south west Western Australia (Howard et al., 2014). This survey also documented a varied range of additional interactions between humans and urban dwelling marsupials in Perth. They indicate the possibility of transfer of infectious agents from humans and pets to marsupials via foodstuffs offered and scavenged, and via direct contact. Similarly, interactions documented here indicate the possibility of transfer of infectious agents from marsupials to humans could occur via various modes of direct contact (such as biting, faecal or urine contamination of living areas, or exposure to marsupial blood via ticks, if the ticks rupture upon

removal) or possibly via ectoparasite inoculation. Similar concerns regarding infection transmission in human-wildlife interaction have been expressed in regards to marsupials and other mammals elsewhere in Australia and overseas (e.g. Eymann et al., 2006; Friend, 2006; Cahill, et al., 2012).

Even in consideration of the survey limitations of a small sample size and non-proportionate sampling, we believe the results suggest a need for a pragmatic, science-driven community education campaign regarding human interactions with cohabiting marsupials, for the benefit of the health of all involved parties. Though recommendations against feeding wildlife are justified and should be upheld, secondary advice regarding appropriate types and quantities of supplemental food, and approaches to supplementary feeding which minimise the potential for harm to marsupials, may be of benefit if some members of the public will feed wildlife irrespective of recommendations. Such secondary advice would help safeguard the health of the marsupials where supplemental feeding is occurring. This is particularly pertinent in the context of the high proportion of overweight or obese quenda trapped in urbanised environments as part of the parasite survey (Chapter 7). Further, as relatively little is known about the zoonotic potential of many infections of native Australian marsupials, and similarly little is known about infectious agents of humans and domestic animals that can infect marsupials, a conservative approach towards biosecurity in wildlife interactions should also be promoted to the public to safeguard the health of all parties. In the absence of informed education on this topic, misleading media-driven "hype" (e.g. Crawford and Mayoh, 2015) becomes the unhelpful, and unacceptable, substitute.

3.5 Acknowledgements

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Chapter 4

Validation of various parasite detection tests for use in the Australian marsupials quenda *(Isoodon obesulus)* and brushtail possums *(Trichosurus vulpecula)*

This chapter is a published article.

Author contributions:

Designed the study: AEH, RCAT

Organised and undertook data collection (field and post-mortem sampling): AEH

Performed the immunofluorescence microscopy of faeces, faecal flotation protocol, gastrointestinal helminth differentiation: AEH

Advised on and provided expert feedback regarding light microscopy and helminth differentiation: ADE

Performed Giardia spp. PCR and sequencing: ALA

Performed Toxoplasma gondii modified agglutination tests and PCR: ARK

Performed statistical analyses: AEH

Wrote and revised the manuscript: AEH

Proofed and critically appraised the manuscript: ALA, ARK, ADE, AJL, IDR, RCAT

4. Validation of various parasite detection tests for use in the Australian marsupials quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*)

4.1   Introduction

In epidemiological investigations of infections in wildlife populations, validation of the test(s) used to detect the infectious agent in the host population under study is required for scientific rigour. Estimates of detection test sensitivity and specificity are essential for data interpretation, and valuable at the study design stage. A lack of validated detection tests is a substantial hindrance to the investigation of infectious disease epidemiology in wildlife worldwide (Stallknecht, 2007), and this is particularly true of parasitic infection epidemiology in Australian marsupial species. The lack of validated tests corresponds to a lack of "gold standard" tests (tests of 100% sensitivity and specificity) against which other tests can be compared in marsupial hosts. This deficiency may be overcome, however, by using Bayesian latent class analysis, which allows for estimation of diagnostic test sensitivity and specificity in the absence of a gold standard (Joseph et al., 1995).

The protozoan parasite *Toxoplasma gondii* is of particular epidemiological interest in Australian marsupial species (Chapter 1.4). The marsupials quenda (syn. southern brown bandicoots, *Isoodon obesulus*) and brushtail possums *(Trichosurus vulpecula)* are susceptible to infection with *T. gondii* (Cook and Pope, 1959; Pope et al., 1957a, b), and may be at particular risk of infection with this parasite due to their common occurrence in urban environments (Hill et al., 2008; Thompson et al., 2009). However, there is not yet a validated test for use in ante mortem identification of the presence of *T. gondii* infection in quenda or brushtail possums, nor in any member of their respective order (Peramelemorphia) or suborder (Phalangeriformes). Similarly, quenda are susceptible to infection with parasites of the genus *Giardia* (Adams et al., 2004; Thompson et al., 2010), which is of epidemiological interest due to the potential

conservation and public health implications (Thompson et al., 2009, 2010). However, no studies have investigated the accuracy of screening tools for detection of *Giardia* spp. infection in this species, nor in any other species within Order Peramelemorphia. Finally, although faecal flotations of helminth eggs are common methodologies used in identifying gastrointestinal helminths present in marsupials sampled ante mortem for epidemiological purposes (e.g. Gerhardt et al., 2000; Turni and Smales, 2001; Robert and Schwanz, 2013), no faecal flotation protocol has been validated in any Australian marsupial species.

We aimed to use Bayesian latent class analysis to validate the use of a number of parasite detection tests in quenda and brushtail possums:

> 1) the modified agglutination test and a PCR protocol in detecting *T. gondii* infection in both host species;
>
> 2) immunofluorescence microscopy and a PCR and sequencing protocol in detecting *Giardia* spp. infection in quenda; and
>
> 3) a faecal flotation protocol in detecting gastrointestinal helminth infections in quenda.

4.2 Methods

4.2.1 Sample collection from quenda and possum carcasses

Sixty-two quenda and 22 brushtail possum carcasses originating from the Statistical Division of Perth (Western Australia) were obtained opportunistically across 2013 - 2014. Sources included a wildlife hospital and animals found dead (e.g. killed by vehicle strike). Seventeen quenda carcasses and one possum carcass were sampled within 72 hours of being found, after storage at 4˚C. Forty-five quenda carcasses and 21 possum carcasses were frozen prior to collection of samples. Frozen carcasses were left to defrost at 4˚C for 12 to 36 hours prior to sample collection (time dependent on body size). Likely cause of death was determined based on clinical signs prior to

death (where carcasses were obtained from the wildlife hospital), circumstances surrounding the death, and gross findings on post mortem examination.

For each animal, sex and whether the animal was an adult or subadult were recorded. Male possums were classified as adult if at least one testis was greater than one cm in width, or subadult if each testis was less than or equal to one cm width. Male quenda were classified as adult if their scrotal width was greater than or equal to two cm, or subadult if their scrotal width was less than two cm (Dr Tony Friend, Department of Parks and Wildlife, pers. comm.). Female possums and quenda were classified as adult if they had a parous pouch, or as subadult if they had a non-parous pouch. In adult females, pouch activity was noted - adult females were classified as having an active pouch if lactating, or an inactive pouch if not lactating.

Where available, the following samples were collected from each carcass:
1) Haemorrhagic / serohaemorrhagic chest fluid: fluid was obtained from the chest cavity, centrifuged at 314 G for 10 minutes, then the serous top layer of the sample (if available) or the top half of the sample (if a serous layer was not available) was transferred to a storage tube and frozen at -20°C until analysis.
2) Organ samples: brain tissue was removed via the foramen magnum and stored in 70% ethanol (at a ratio of at most 1:4), and 2 mL of heart, liver, tongue and diaphragm tissue (or the whole organ, if total volume was lower) was cut to approximately 1 cm width and added to separate tubes of 8 mL 70% ethanol. All organ samples were stored at 4°C until analysis.
3) Faecal material obtained from the large intestine (quenda only): 2 mL faeces were added to 8 mL 10% buffered formalin and thoroughly mixed. One mL faeces were added to 8 mL 70% ethanol and thoroughly mixed. Samples were stored at 4°C until analysis.
4) Gastrointestinal helminths (quenda only): the entire gastrointestinal tract and its contents were screened for helminths under a dissection microscope. After the faecal

flotations were completed, helminths were also recovered from the sample of faecal matter used for the flotation. All observed nematodes were removed and stored in 70% ethanol. Cestodes were removed and stored in 10% buffered formalin. Helminths were stored at 4˚C until analysis.

4.2.2　　　　Sample collection from free-ranging quenda

Faecal samples were also obtained from quenda trapped as part of a parasite survey in the Statistical Division of Perth. Two mL faeces were added to 8 mL 10% buffered formalin and thoroughly mixed. One mL faeces were added to 8 mL 70% ethanol and thoroughly mixed. Samples were stored at 4˚C until analysis. Trapped quenda had age, sex and pouch activity recorded, as described for the quenda carcasses.

4.2.3　　　　Sample collection permits

All samples were obtained under Murdoch University Animal Ethics Permit R2530/12, and Department of Parks and Wildlife Regulation 17 (SF009640) and Regulation 4 (CE004287) permits.

4.2.4　　　　*Toxoplasma gondii* test evaluation in quenda and brushtail possums

Chest fluid samples from quenda and possum carcasses were tested using a commercial modified agglutination test kit (Toxo-Screen DA, bioMérieux, France), according to manufacturer's instructions. The animal was considered to have tested positive on the modified agglutination test if it was positive at titres of 1:40 and / or 1:4000. The same observer performed all these tests.

For the *T. gondii* PCR, DNA was extracted from tissue samples using a blood and tissue kit (QIAGEN GmbH, Hilden, Germany), with some variations. A freeze / thaw step was conducted prior to addition of proteinase K, to facilitate disruption of *T. gondii*

tissue cysts. Tissue was macerated in 50 µL PBS in a screw cap tube, 180 µL buffer ATL was added, and tubes were subjected to 8 cycles of alternating between one minute in liquid nitrogen and one minute in a 95°C waterbath. Proteinase K was added, samples were incubated overnight at 56°C, and the protocol was then continued according to the manufacturer's instructions, with a final elution volume of 50 µL. Eluted DNA was stored at -20°C until required for PCR. A positive control (*T. gondii* infected quenda liver) and negative control (phosphate buffered saline) were included in each batch of DNA extractions.

The target for PCR amplification of *T. gondii* DNA was the B1 gene, using a nested PCR with primers: external forward 5'-TGTTCTGTCCTATCGCAACG; external reverse 5'-ACGGATGCAGTTCCTTTCTG; internal forward 5'-TCTTCCCAGACGTGGATTTC; internal reverse 5'-CTCGACAATACGCTGCTTGA (Grigg and Boothroyd, 2001). Each reaction of the nested PCR was performed in a 25 µL volume consisting of 1-2 µL extracted DNA, 1.5 mM MgCl$_2$, 1 x reaction buffer (Fisher Biotec, Perth, Australia), 200 µM of each dNTP, 0.4 µM of each appropriate forward and reverse primer, and 0.2 units of Taq DNA polymerase (Fisher Biotec, Perth, Australia) in a cresol loading dye solution. PCR was performed in a thermocycler (BioRad, Gladesville, Australia) with the following conditions: 95°C for 1 minute, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by a final extension of 72°C for 5 minutes. Each PCR plate contained a positive control (purified DNA from cultured *T. gondii* tachyzoites) and negative controls. PCR products were either used immediately, or stored at 4°C until required for either the internal PCR or visualisation. After the internal PCR amplification, PCR products were separated on a 2% agarose gel containing SYBRsafe DNA stain (Molecular Probes Inc., Eugene, USA) by electrophoresis, and gels were photographed under ultraviolet light. Carcasses were considered to have tested positive by PCR if specific DNA bands of the correct product size for *T. gondii* were amplified from any or all of the brain, heart, diaphragm, liver or tongue. The same observer performed all PCR tests.

Of the non-specific DNA bands that were sometimes amplified by PCR (faint bands that were not the correct product size for *T. gondii*), the ten non-specific bands that were closest to the size of *T. gondii* were sequenced to check that *T. gondii* had not been amplified. These DNA samples were purified using a purification system (Agencourt AMPure XP, Beckman Coulter, Beverley, USA), following the manufacturer's instructions. Samples were sequenced in both directions using a sequencing kit (ABI Prism™ Terminator Cycle Sequencing kit, Applied Bio-systems, California, USA) on a DNA analyser (Applied Biosystems 3730 DNA Analyzer, Thermo Fisher Scientific, Waltham, USA). Sequences were analysed using Geneious 7.2. (Kearse et al., 2012).

4.2.5 *Giardia* spp. test evaluation in quenda

Immunofluorescence microscopy was used to identify *Giardia* spp. cysts in faecal samples obtained from trapped quenda and quenda carcasses, using Merifluor Cryptosporidium / Giardia kits (Meridian Bioscience, Inc., Cincinnati, USA), according to manufacturer's instructions for a non-concentrated sample. Samples were considered positive on immunofluorescence microscopy if at least one cyst of appropriate fluorescence and *Giardia* spp. morphology was identified. The same observer performed all immunofluorescence microscopy tests.

PCR and sequencing was undertaken on all immunofluorescence microscopy positive samples and a random sample of 10 immunofluorescence microscopy negative samples. Faecal samples preserved in 70% ethanol had DNA extracted for amplification of *Giardia* spp. at three loci - 18S rRNA, ITS1-5.8s-ITS2 and *gdh* - and all amplified products on PCR were subject to sequencing, as described in Chapter 9.2.4. Quenda were considered positive for *Giardia* spp. via this PCR and sequencing protocol if genetic material was amplified, and amplified product sequenced as *Giardia* spp., at one or more loci. The same observer performed all PCR work.

As an addendum to the *Giardia* spp. validation, during the faecal flotations for identification of gastrointestinal helminth eggs (described in 4.2.6) the microscopist assessed the sample for the presence of *Giardia* spp. cysts.

4.2.6   Evaluation of a faecal flotation protocol as an indicator of gastrointestinal helminth infections in quenda

For the faecal flotation protocol, each formalin-preserved quenda faecal sample originating from a carcass was centrifuged at 850G for 2 minutes, with the formalin supernatant discarded. The sample was then divided between two 10 mL centrifuge tubes. The samples were re-homogenised in 9 mL distilled water, and centrifuged at 850G for 2 minutes, with the supernatant discarded. One tube was analysed using zinc sulphate flotation. The faecal matter was thoroughly mixed with 9 mL zinc sulphate solution (SG 1.20), then centrifuged at 850 G for 2 minutes. A flamed wire loop was used to transfer material from the surface of the flotation to a slide, and a coverslip was added. Slides were systematically scanned at 200x magnification using a BX50 microscope (Olympus, Tokyo, Japan). The second tube was analysed using sodium nitrate flotation. The faecal matter was thoroughly mixed with 9 mL sodium nitrate solution (SG 1.37) and centrifuged at 850 G for 2 minutes. The tube was then topped up with sodium nitrate solution to create a meniscus. A coverslip was placed on top of the tube and left for 10 minutes, then the coverslip was placed on a slide and systematically scanned at 200x magnification using a BX50 microscope. Before the first sodium nitrate slide was examined, the sodium nitrate tube was topped up with sodium nitrate solution again to create another meniscus, and a second coverslip was placed on top. This was transferred to a slide and examined at 100x magnification using a BX50 microscope, directly after the first sodium nitrate flotation slide had been examined (a pilot study indicated that examining a second slide from the sodium nitrate tube substantially increased the likelihood of detecting *Trichuris* spp. eggs in quenda faeces (A. Hillman - unpublished data).

Helminth eggs were identified morphologically, using features and size ranges described in the literature (Mawson, 1960; Smales, 1997; Vaucher and Beveridge, 1997) and in microscopic photography records collated from prior quenda faecal analyses (A. Elliot - unpublished data; see also Appendix A). Quenda were considered positive for the respective helminth type via the faecal flotation protocol if at least one egg of appropriate morphological features was identified on any or all of the three faecal flotations performed as part of the faecal flotation protocol. All faecal flotations were undertaken by the same observer.

The gastrointestinal helminths obtained from the quenda carcasses via the microscopic gastrointestinal tract helminth screen were differentiated to genus, family or infraorder level using a BX50 microscope (at four to 200x magnification, depending on the worm genus / family / infraorder), using published quenda helminth descriptions (Mawson, 1960; Smales, 1997; Vaucher and Beveridge, 1997). Where cestodes were too degraded to have retained identifying features (particularly rostellar hooks), the morphological features of eggs obtained from gravid proglottids were used to identify the genus of cestode. If gravid proglottids were not present, and thus the cestode was not identifiable morphologically, the sample was excluded from cestode sensitivity and specificity calculations. Quenda were considered to have tested positive for the respective nematode types on microscopic gastrointestinal tract examination if at least one nematode of the respective morphological type was identified on gastrointestinal tract screening. Quenda were considered to have tested positive for a cestode genus on microscopic gastrointestinal tract examination if at least one cestode of that morphological type was obtained and identified, or if eggs of that cestode genus were obtained and identified from gravid proglottids of a cestode that was not otherwise morphologically identifiable. All gastrointestinal helminth identifications were undertaken by the same observer.

4.2.7 Statistical analysis

The sensitivity and specificity of the parasite detection tests were calculated via Bayesian latent class analysis, using R v3.1.1 (The R Project for Statistical Computing, 2015), with interface BayesLatentClassModels v1.13 (Dendukuri et al., 2015). Briefly, in the Bayesian latent class analyses used in this study (where two detection test results are available per animal, with tested animals obtained from one population), five parameters require consideration - infection prevalence, and the sensitivity and specificity of each test. Parameters are entered as either informative or non-informative distributions. As the number of parameters exceeds the three degrees of freedom provided by the data, informative prior distribution inputs are required for at least two of the five model parameters for model identifiability. Published data (where available) and / or expert knowledge can be used to guide informative (beta) prior distribution inputs. Non-informative (uniform) prior distributions can be used where no information is available to guide prior distribution estimations. The prior distribution inputs are then modelled with the detection test data obtained from the study and the unknown latent variable (representing the true infection status of the host) using the iterative Gibbs Sampler Markov-chain Monte Carlo method, to obtain posterior distribution estimates of the sensitivity and specificity of each detection test and of the infection prevalence (though in this study, prevalence is not of interest as an output) (Joseph et al., 1995).

For the Bayesian latent class analysis of the *T. gondii* tests, tests were modelled as conditionally independent. The prior distribution inputs were identical for the quenda and brushtail possum models, and are described in Table 4.1. The relatively conservative prior distribution for modified agglutination test specificity (0.50 - 0.99) was based on findings from several macropod species (Family Macropodidae) – an ELISA was validated as highly specific, and this ELISA was shown to have excellent agreement with the modified agglutination test (Parameswaran, 2008; Parameswaran et al., 2009). The more precise prior distribution used for PCR specificity was based on previous research findings at the B1 locus (Table 4.1).

For the Bayesian latent class analysis of the *Giardia* spp. tests, tests were modelled as conditionally independent, as the widely reported high sensitivity and specificity of *Giardia* spp. detection via immunofluorescence microscopy of faeces in other species indicated that conditional dependence between tests was unlikely to impact these models. The prior distribution inputs for the *Giardia* spp. analysis are described in Table 4.1. The relatively conservative informative prior distribution used for PCR and sequencing sensitivity was chosen in view of the variable results observed via PCR in different studies of *Giardia* spp. that used varying protocols on a variety of host species (Thompson and Ash, 2015). The high and narrow prior distribution used for PCR and sequencing specificity was selected as the use of sequencing in our case definition made false positive results highly unlikely.

For the Bayesian latent class analysis of the gastrointestinal helminth tests, tests were modelled as conditionally independent. The prior distribution inputs for the gastrointestinal helminth analyses are described in Table 4.1. All informative priors were based on expert opinion, obtained in consulting expert personnel involved in past and current marsupial parasitology at Murdoch University. The informative prior distributions used for the specificity of the faecal flotation protocol in detection of *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp. and *Potorolepis* spp. were recommended based on the distinctive morphological features of these helminth eggs rendering false positives unlikely. The prior distribution for faecal flotation specificity for the detection of strongyles and strongyloids was recommended in consideration of the possibility that parasite eggs of quenda prey items may be present in the quenda faeces, and there are strongyle and strongyloid helminths with similar eggs parasitic in a wide range of species. The prior distribution for faecal flotation specificity for the detection of *Trichuris* spp. infection was recommended in consideration of the risk of eggs of other capillarids (Superfamily Trichinelloidea, e.g. *Capillaria* spp.) being mistaken for those of *Trichuris* spp. The prior distributions for the sensitivities of the observer finding *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp. and

Potorolepis spp. helminths in the gastrointestinal tract of infected quenda and correctly differentiating them to genus level were recommended based on the relatively large size of adult helminths, and the reasonably uncomplicated taxonomic differentiation required, rendering false negatives relatively unlikely. Uninformative prior distributions were used for sensitivity of detection and differentiation of strongyles (Infraorder Rhabditomorphia), strongyloids (Family Strongyloididae) and *Trichuris* spp., based on the tiny size (strongyles and strongyloids) and the tiny (coiled) size and apparent fragility of *Trichuris* spp. worms after recovery from frozen carcasses, causing uncertainty about the potential for false negatives. The informative prior distribution inputs for the specificity of the observer finding helminths in the quenda gastrointestinal tract and correctly differentiating to a genus / family / infraorder level were recommended based on the distinctiveness of the helminth morphology at the taxonomic level of differentiation required, rendering false positives unlikely.

The number of Gibbs Sampler iterations for each model started at 10 500 iterations, with the first 500 "burn in" iterations dropped from the results. The number of iterations was doubled if the model did not adequately converge using the lower number of iterations (Table 4.1).

Table 4.1 Prior distribution inputs for Bayesian latent class analysis of parasite detection test validity in quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*)

Test validation: parasite (host)	Prior distributions	Prior distribution inputs	Informative prior distribution references/ expert opinion[1]	No. Gibbs Sampler iterations[2]
Toxoplasma gondii (quenda and possums)	Prevalence	Non-informative	-	21 000
	Modified agglutination test (MAT) sensitivity	Non-informative	-	
	MAT specificity	0.50 – 0.99	Parameswaran, 2008; Parameswaran et al., 2009	
	PCR sensitivity	Non-informative	-	
	PCR specificity	0.95 – 0.99	Burg et al., 1989; Guy et al., 1996; Pelloux et al., 1998; Robert-Gangneux et al., 1999; Romand et al., 2001.	
Giardia spp. (quenda)	Prevalence	Non-informative	-	10 500
	Immunofluorescence microscopy (IMF) sensitivity	0.8 – 0.99	Garcia et al., 1992; Azia et al., 2001; Geurden et al., 2008; Rishniw et al., 2010; Sommer et al., 2015.	
	IMF specificity	0.9 – 0.99		
	PCR & sequencing sensitivity	0.6 – 0.99	Miller & Sterling, 2007; Beck et al., 2011a; Beck et al., 2011b; McDowall et al., 2011; Sommer et al., 2015.	
	PCR & sequencing specificity	0.95 – 0.99		
Strongyles[3], strongyloids[4] (quenda)	Prevalence	Non-informative	-	21 000
	Faecal flotation protocol (FFP) sensitivity	Non-informative	-	
	FFP specificity	0.8 – 0.99	Expert opinion	
	GI (gastrointestinal) helminth screen sensitivity	Non-informative	-	
	GI helminth screen specificity	0.95 – 0.99	Expert opinion	
Labiobulura spp., *Linstowinema* spp., *Physaloptera* spp., *Potorolepis* spp. (quenda)	Prevalence	Non-informative	-	21 000 (*Physaloptera* spp. only) 10 500 (all other taxa)
	FFP sensitivity	Non-informative	-	
	FFP specificity	0.9 – 0.99	Expert opinion	
	GI helminth screen sensitivity	0.8 – 0.99	Expert opinion	
	GI helminth screen specificity	0.95 – 0.99	Expert opinion	
Trichuris spp. (quenda)	Prevalence	Non-informative	-	10 500
	FFP sensitivity	Non-informative	-	
	FFP specificity	0.5 – 0.99	Expert opinion	
	GI helminth screen sensitivity	Non-informative	-	
	GI helminth screen specificity	0.95 – 0.99	Expert opinion	

[1] Expert opinion was obtained by consulting experts involved in past and current marsupial parasitology at Murdoch University, Australia.

[2] Where 21 000 iterations were used, the first 1000 "burn in" iterations were dropped from results; where 10 500 iterations were used, the first 500 "burn in" iterations were dropped from results.

[3] Infraorder Rhabditomorphia.

[4] Family Strongyloididae.

Model validity for each infection type was established in a number of ways. Firstly, Gibbs sampler trace plots were evaluated to ensure convergence, and models were rerun with different starting values five times to ensure consistency of output. Secondly, the observed *vs.* the median predicted test profiles were compared to ensure they were similar. Finally, the observed and expected probabilities of agreement between the tests were compared to ensure values were similar, and the associated probability statistic was between 0.05 and 0.95.

The median value of the modelled test sensitivities and specificities were reported as the point estimates, with 95% credible intervals (CrI) (credible intervals are Bayesian analogues of frequentist confidence intervals). The prevalence outputs, and the output of the sensitivity and specificity of the observer finding and correctly identifying helminths to a genus / family / infraorder level were not reported. This was because this study was not designed to measure infection prevalences, and though the data provided by the observer obtaining and differentiating helminths were a valuable component of the latent class analysis, the sensitivity and specificity of this test is not of practical value of itself to future research.

Sensitivity analyses of all models were undertaken, by varying each informative prior distribution separately, while all other model inputs were held constant. The credible limits of the informative priors were decreased by 10% and 20% (gross), respectively. A change in the sensitivity and / or specificity point estimate by 5% (gross) or more was considered 'substantial', and reported in the results.

4.3 Results

4.3.1 Likely cause of death of carcasses used in this study

Of quenda carcasses used in this study, 54 / 62 (87.1%) were judged to have been killed by vehicle strike, attack by another animal, household poisons (such as rat and snail baits) or drowning in backyard pools or ponds. Of possum carcasses used in this study, 15 / 22 (68.2%) were judged to have been killed by traumatic injuries (particularly vehicle strike or attack by another animal), electrocution or household poisons (such as rat and snail baits).

4.3.2 *Toxoplasma gondii* test evaluation in quenda and brushtail possums

Chest fluid and body tissues were available from 56 quenda carcasses and 22 brushtail possum carcasses. The quenda carcasses comprised six subadult females, 12 adult females, 21 subadult males, 16 adult males, and an additional subadult quenda of indeterminate sex. Of the adult female quenda, nine had an active pouch, and three had an inactive pouch. The possum carcasses comprised two subadult females, nine adult females, four subadult males and seven adult males. Of the adult female possums, two had an active pouch, and seven had an inactive pouch.

All five targeted body tissues (brain, heart, liver, diaphragm and tongue) were available from 51 quenda carcasses and all brushtail possum carcasses. Due to traumatic injuries, diaphragmatic tissue was not recoverable from three quenda, heart tissue was not recoverable from one quenda, and sampling of brain tissue was severely compromised in one quenda. All carcasses positive on the modified agglutination test had all five tissues available for testing.

The *Toxoplasma gondii* tests in brushtail possums were 95.5% concordant (21 / 22 possums). One possum was positive on the modified agglutination test, whilst bands

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specific for *T. gondii* were not amplified from any brushtail possum tissues. In quenda, the *Toxoplasma gondii* tests were 92.9% concordant (52 / 56 quenda). Four quenda were positive on the modified agglutination test, whilst bands specific for *T. gondii* were not amplified from any quenda tissues (Table 4.2). The Bayesian sensitivity and specificity estimates of the modified agglutination test and the PCR protocol are shown in Table 4.3. On sensitivity analysis, no substantial changes occurred to the point estimates of sensitivity and specificity of either *T. gondii* test in either species.

Table 4.2 Cross tabulations of the results of the modified agglutination test (MAT) and PCR protocol in detecting *Toxoplasma gondii* infections in quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*)

Host			PCR	
			Positive	Negative
Brushtail possums	**Modified agglutination test**	Positive	0	1
		Negative	0	21
Quenda	**Modified agglutination test**	Positive	0	4
		Negative	0	52

The ten non-specific bands amplified by *T. gondii* PCR that were closest to the expected size for *T. gondii* (from ten tissues obtained from six quenda) did not sequence as *T. gondii.*

One of the 22 possums and one of the 56 quenda were known to have non-specific neurological symptoms prior to death. Both animals were negative on both the modified agglutination test and PCR.

Table 4.3 Sensitivity and specificity of various parasite detection tests in quenda (*Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*)

Host species	Parasite	Parasite detection test	Sensitivity median (95% CrI[1])	Specificity median (95% CrI[1])
Brushtail possums	*Toxoplasma gondii*	Modified agglutination test	31.5% (1.2 - 95.5%)	94.2% (78.0 - 99.3%)
		PCR protocol	22.0% (0.5 - 93.6%)	97.8% (95.4 - 99.1%)
Quenda	*Toxoplasma gondii*	Modified agglutination test	30.2% (1.6 - 95.4%)	93.2% (78.5 - 98.9%)
		PCR protocol	12.8% (0.3 - 90.0%)	98.0% (95.8 - 99.1%)
Quenda	*Giardia* spp.	Immunofluorescence microscopy	97.5% (93.4 - 99.4%)	96.4% (89.9 - 99.0%)
		PCR and sequencing protocol	68.8% (59.9 - 76.8%)	97.7% (95.1 - 99.0%)
Quenda	Strongyles[2]	Faecal flotation protocol	96.5% (82.6 - 99.9%)	94.2% (79.7 - 99.0%)
	Labiobulura spp.		74.2% (58.4 - 87.0%)	97.0% (91.8 - 99.1%)
	Linstowinema spp.		81.4% (65.7 - 92.4%)	96.4% (90.2 - 99.0%)
	Physaloptera spp.		51.0% (2.7 - 97.5%)	95.6% (90.8 - 98.2%)
	Strongyloids[3]		17.0% (0.7 - 88.3%)	95.7% (88.2 - 99.0%)
	Trichuris spp.		91.3% (63.5 - 99.6%)	88.0% (71.3 - 98.8%)
	Potorolepis spp.		36.1% (13.8 - 66.5%)	97.5% (93.4 - 99.3%)

[1] CrI= credible interval

[2] Infraorder Rhabditomorphia

[3] Family Strongyloididae

4.3.3   *Giardia* spp. test evaluation in quenda

Faecal samples available for the *Giardia* spp. test validation comprised samples from 110 immunofluorescence microscopy-positive quenda, and ten immunofluorescence microscopy-negative quenda. The samples were obtained from six subadult females, 42 adult females, 11 subadult males and 61 adult males. Of the adult females, 33 had an active pouch, and nine had an inactive pouch. Eleven samples were from quenda carcasses, with 109 samples obtained from trapped quenda.

The *Giardia* spp. infection detection test results were concordant in 83 / 120 (69.2%) quenda (Table 4.4). Thirty-six quenda were positive for *Giardia* spp. infection on immunofluorescence microscopy, but negative by PCR and sequencing. *Giardia* spp. cysts were identified in 22 (61.1%) of the immunofluorescence microscopy-positive / PCR and sequencing-negative quenda, by light microscopy during the faecal flotations.

Table 4.4 Cross tabulation of the results of immunofluorescence microscopy and a PCR and sequencing protocol in detecting *Giardia* spp. infections in quenda (*Isoodon obesulus*)

		Immunofluorescence microscopy	
		Positive	Negative
PCR and sequencing protocol	Positive	74	1
	Negative	36	9

Bayesian sensitivity and specificity estimates of the *Giardia* spp. tests are presented in Table 4.3. On sensitivity analysis, no substantial changes occurred to the point estimates of sensitivity and specificity of both *Giardia* spp. tests.

4.3.4 Evaluation of a faecal flotation protocol as an indicator of gastrointestinal helminth infections in quenda

Gastrointestinal tract helminth screens and faecal flotation analyses were available from 49 quenda carcasses. This comprised four subadult females, 11 adult females, 19 subadult males and 15 adult males. Of the adult female quenda, two had an inactive pouch and nine had an active pouch.

Six nematode taxa, consisting of strongyles (Infraorder Rhabditomorphia), *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp., strongyloids (Family Strongyloididae) and *Trichuris* spp., were detected by the faecal flotation protocol and / or the microscopic gastrointestinal helminth screen. The cestode genus *Potorolepis* was detected by the faecal flotation protocol and the microscopic gastrointestinal helminth screen. Three

quenda carcasses contained unidentifiable cestode segments and were thus excluded

from *Potorolepis* spp. results, and the *Potorolepis* spp. sensitivity and specificity

calculations. Cestode eggs were not identified in the faeces of any of these three

quenda.

The concordance of the gastrointestinal helminth detection tests were: 34 / 49 (69.4%)

for strongyles; 40 / 49 (81.6%) for *Labiobulura* spp.; 41 / 49 (83.7%) for *Linstowinema*

spp.; 46 / 49 (93.9%) for *Physaloptera* spp.; 44 / 49 (89.8%) for strongyloids; 40 / 49

(81.6%) for *Trichuris* spp.; and 38 / 46 (82.6%) for *Potorolepis* spp. (Table 4.5). The

Bayesian sensitivity and specificity estimates of the faecal flotation protocol in

identifying infection with various gastrointestinal helminths are summarised in Table

4.3.

Table 4.5 Cross tabulations of the results of a faecal flotation protocol (FFP) and microscopic examination of the gastrointestinal (GI) tract in detecting gastrointestinal helminth infections in quenda (*Isoodon obesulus*)

Helminth type			Microscopic GI tract exam:	
			Positive	Negative
Strongyles[1]	**FFP:**	Positive	20	15
		Negative	0	14
Labiobulura spp.	**FFP:**	Positive	27	0
		Negative	9	13
Linstowinema spp.	**FFP:**	Positive	27	2
		Negative	6	14
Physaloptera spp.	**FFP:**	Positive	0	3
		Negative	0	46
Strongyloids[2]	**FFP:**	Positive	0	2
		Negative	3	44
Trichuris spp.	**FFP:**	Positive	8	9
		Negative	0	32
Potorolepis spp.	**FFP:**	Positive	4	0
		Negative	8	34

[1] Infraorder Rhabditomorphia

[2] Family Strongyloididae

On sensitivity analysis, no substantial changes occurred to the point estimates of

sensitivity and specificity of the faecal flotation protocol in detecting *Labiobulura* spp.,

Linstowinema spp., *Physaloptera* spp., strongyloids and *Trichuris* spp. When the lower

credible limit of the informative prior distribution for the specificity of the faecal flotation

protocol in identifying strongyle infections was extended to 0.6, the point estimate of specificity of the faecal flotation protocol in detecting strongyle infections decreased to 88.2% (95% CrI 57.3 – 98.8%). When the lower credible limit of the informative prior distribution of the specificity of the microscopic gastrointestinal tract exam in identifying *Potorolepis* spp. infections in quenda was widened to 0.75, the point estimate for the sensitivity of detecting *Potorolepis* spp. via the faecal flotation protocol increased to 45.2% (95% CrI 16.3 – 92.1%).

4.4   Discussion

This study presents the first validation data regarding tests for use in the detection of *Giardia* spp. and gastrointestinal helminth infections in any Australian marsupial species (Superorder Australidelphia), and the first validation data for tests to detect *T. gondii* infection in any species of Order Peramelemorphia and Suborder Phalangeriformes.

The robustness of the Bayesian models on sensitivity analysis suggests that the results were primarily driven by the data, rather than unduly influenced by the choice of credible limits for the informative prior distributions. Substantial changes to the point estimates of sensitivity or specificity only occurred when informative prior distribution limits that were considered highly implausible were used.

The apparently accidental cause of death of the majority of carcasses used in the study may have avoided substantial bias of results towards that of "sickly" animals, therefore maintaining the applicability of the results to epidemiological studies in the broader host populations.

4.4.1 *Toxoplasma gondii* test evaluation in quenda and brushtail possums

Our findings suggest that both the modified agglutination test (at the titres used in this study) and the PCR protocol are highly specific in the detection of *T. gondii* infection in quenda and brushtail possums. This study used chest fluid for the modified agglutination test, rather than serum, which may bias findings if extrapolating these results to the use of serum. However, findings of a study comparing *T. gondii* ELISA results in tissue fluid *vs.* serum did not suggest ELISA specificity was substantially affected when using tissue fluid (Hill et al., 2006). The finding of high modified agglutination test specificity in quenda and brushtail possums concurs with findings in other host species (Dubey et al., 1997; Mainar-Jaime and Barberán, 2007; Györke et al., 2011).

The data were insufficient to assess the sensitivity of these tests in an adequately precise manner, as the apparent prevalence (using both tests) of *T. gondii* was low in both the quenda and possum study samples.

4.4.2 *Giardia* spp. test evaluation in quenda

This study found both immunofluorescence microscopy and the PCR and sequencing protocol to be highly specific in the detection of *Giardia* spp. infections in quenda. This finding concurs with high specificities observed in other host species (Garcia et al., 1992; Nantavisai et al., 2007; Geurden et al., 2008).

Immunofluorescence microscopy was highly sensitive in detecting *Giardia* spp. infection in quenda faeces, and substantially more so than the PCR and sequencing protocol. This reflects other research findings, where amplification by PCR was not successful in all *Giardia* spp. positive faecal samples tested (McDowall et al., 2011; Pallant et al., 2015). The identification of *Giardia* spp. cysts by faecal flotations in the

majority of the immunofluorescence microscopy-positive, PCR and sequencing-negative faecal samples, provided reassurance that the discrepancy between the number of samples positive by immunofluorescence microscopy *vs.* those positive by the PCR and sequencing protocol was not due to a lack of specificity by immunofluorescence microscopy. As *Giardia* spp. detection by faecal flotations has been shown to lack sensitivity in other species (e.g. Geurden et al., 2008; Rishniw et al., 2010), the fact that *Giardia* spp. cysts were not identified in all flotations is not unexpected. The sensitivity of both immunofluorescence microscopy and PCR and sequencing in detecting *Giardia* spp. infection in quenda may have been overestimated if there are commonly periods in which infected quenda do not shed any cysts in their faeces.

As only two faecal samples tested positive for *Giardia* spp. by PCR and sequencing at the *gdh* locus, and these samples also tested positive for *Giardia* spp. at the two other loci tested, the *gdh* locus could be excluded from the PCR and sequencing protocol with no change in protocol sensitivity or specificity (further detail of the PCR and sequencing results at the individual loci are presented in Chapter 9.3).

4.4.3   Evaluating a faecal flotation protocol as an indicator of gastrointestinal helminth infections in quenda

The specificities of detection of various gastrointestinal helminth infections by the faecal flotation protocol were generally high. The faecal flotation protocol specificities for *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp. and *Potorolepis* spp. may have been biased downwards as we chose to be relatively conservative in inputting prior distributions, despite the reasonable expectation of a very high degree of specificity in the protocol for these helminths (distinctive helminth egg morphology, with all flotations performed by an experienced observer). However, we cannot exclude the possibility that these specificities reflect a small degree of misclassification. The

relatively lower specificity for *Trichuris* spp. may have been attributable to eggs of other capillarids being mistaken for those of *Trichuris* spp. - if this is the case, our observations on microscopy suggest that there is very little difference in the morphology or size range of eggs between *Trichuris* spp. and other capillarid genera in quenda. The published description of smooth-shelled eggs of a size range of 65 x 35 μm for *T. peramelis* (Mawson, 1960) was not commonly reflected in the *Trichuris* spp. eggs frequently observed in the faeces of quenda in which *Trichuris* spp. worms were obtained from their gastrointestinal tract, and in the *Trichuris* spp. worms themselves. Eggs observed in this study did not have smooth shells and were typically 55 - 57.5 μm x 22.5 - 25 μm - only occasionally larger or smaller (A. Hillman - unpublished data).

The faecal flotation protocol detected the presence of the helminths *Labiobulura* spp., *Linstowinema* spp., *Trichuris* spp. and strongyles in quenda with a moderate to high degree of sensitivity, though results are limited by a lack of precision. The imperfect sensitivity of the faecal flotation protocol in detecting gastrointestinal helminth infections may be influenced by factors such as prepatent infections (particularly given the relatively large number of subadult quenda comprising the study sample) and intermittent shedding of eggs. Although limited by a lack of precision, the relatively low sensitivity of the faecal flotation protocol in detecting the cestode genus *Potorolepis* spp., reflects a lack of sensitivity in the detection of other intestinal cestodes via similar methods in other host species (e.g. Nilsson et al., 1995; Martinez-Carrasco et al., 2007; Liccioli et al., 2012). Our point estimate of 36.1% sensitivity may have been an overestimate if any or all of the three unidentifiable cestode-positive quenda were infected with *Potorolepis* spp., which is considered highly likely. *Physaloptera* spp. and strongyloids were not sufficiently prevalent in our study to provide an adequately precise estimate of faecal flotation protocol sensitivity.

Compared to our findings, both the sensitivity and specificity of faecal flotations may be influenced through the use of different observers, or if the particular faecal flotation protocol varies to that utilised in this study.

4.5   Conclusions

The tests investigated in this study all had high specificities (point estimates > 88%). Immunofluorescence microscopy was highly sensitive in the detection of *Giardia* spp. in quenda, and is a more appropriate screening test for *Giardia* spp. infection than the tested PCR and sequencing protocol in this host species. Though the faecal flotation protocol sensitivities were limited in general by a lack of precision, findings indicate that *Potorolepis* spp. infection prevalence is likely to be substantially underestimated using this test, and analytical extrapolations considering this parasite would have reduced power.

4.6   Acknowledgements

4.7 References

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Chapter 5

Biosecurity and cross-contamination in epidemiological studies involving trapping and sampling Australian marsupial species

This chapter is intended for publication.

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5. Biosecurity and cross-contamination in epidemiological studies involving trapping and sampling Australian marsupial species

5.1 Introduction

Trapping and subsequently sampling animals is an integral part of research involving wildlife. However, this entails biosecurity risks that potentially affect animal and human welfare due to anthropozoonoses and anthropogenic spread of infections within and between wildlife populations (Bosch et al., 2013; Messenger et al., 2014). Additionally, poorly managed biosecurity in trapping may result in cross-contamination of samples from different animals, which may cause substantial misclassification bias or measurement error in epidemiological studies dependent on trapping for sample collection. Such bias may substantially impact prevalence estimates, and lead to errors in interpretation of data.

Thus, biosecurity in trapping and sampling methodology, and the potential impact of cross-contamination on data obtained from trapping, require consideration. The purpose of this paper is to describe practical approaches to biosecurity in trapping and sampling small marsupials, and approaches to evaluating data for potential impact of cross-contamination, using our own study of quenda (syn. southern brown bandicoots, *Isoodon obesulus*) and brushtail possums (*Trichosurus vulpecula*) as an example (which was undertaken under Murdoch University Animal Ethics Permit R2530/12).

5.2 The potential for cross-contamination of samples in trapping small marsupials

Trapping of small marsupials typically occurs across multiple days and / or nights in a particular location. Animals may be in traps overnight, and defaecate repeatedly. If faecal samples collected from traps are contaminated with faeces of animals trapped

previously, results of faecal analyses for that animal may not be accurate. This may be particularly the case if samples are screened with highly sensitive molecular / PCR based tests (Gasser, 2006).

In sampling small marsupials, cloth or hessian handling bags are typically deployed to restrain the animal. Handling bags may become contaminated by an animal's ectoparasites, and sometimes by faeces. For example, in our study, ectoparasites were present in the majority of handling bags used for quenda, and faeces were commonly found in the bags used for brushtail possums. Ectoparasites documented on an animal as a result of exposure via a contaminated handling bag will not provide data that represents the true epidemiology of ectoparasitism within that population. Cross-contamination of faecal samples could have the same effect where faecal-borne pathogens are of interest.

5.3 General hygiene principles around which biosecurity practices can be designed

Traps, handling bags, animal handlers and equipment all required consideration, and the following principles guided our practices.

5.3.1 Traps

In the field, traps can be readily cleaned, using paper towels and a broad-spectrum bactericidal, virucidal and fungicidal disinfectant spray to remove organic matter from the trap after an animal has occupied it. The disinfectant can then be sprayed around the trap and left for at least the minimum contact time recommended by the manufacturer before the trap is reopened. Contact time varies depending on the product used- whilst manufacturer recommendations often indicate a five to ten minute contact time to disinfect for a wide range of pathogens, with up to half an hour required for more resistant viruses, provided organic matter has been removed prior to

disinfection (e.g. Health and Hygiene, 2016), contact times of up to one minute often provide substantial reductions in microbial contamination (Centers for Disease Control and Prevention, 2009).

Placing traps on newspaper facilitates collection of faecal samples and helps minimise ground contamination with faeces. If the newspaper is disturbed and there is substantial ground contamination with faeces, the traps can be moved to an adjacent suitable location. This will likely decrease the risk of cross-contamination of faecal samples. Newspaper requires changing after an animal has occupied the trap.

Between trapping sites, traps can be thoroughly scrubbed to remove all organic matter, soaked in broad-spectrum bactericidal, virucidal and fungicidal disinfectant for the recommended contact time, and dried. This is likely to minimise the risk of spread of infection and cross-contamination between sites.

5.3.2   Handling bags

Using a clean handling bag for each animal is readily achievable. Used handling bags can be cleared of ectoparasites and faeces, and then thoroughly laundered and dried, prior to use with another animal.

5.3.3   Animal handlers

The risk of cross-contamination of samples - as well as transfer of many infections between animals, or between personnel and animals - is likely to be substantially reduced if personnel directly handling animals wear disposable gloves, and change gloves between animals - as per human clinical settings (Leclair et al., 1987; Olsen et al., 1993; Tenorio et al., 2001). Hand sanitiser - readily available in field friendly, alcohol based preparations - is a valuable adjunct to a rigorous glove use protocol, though is not adequate as an exclusive hand-hygiene measure (Boyce and Pittet,

2002), particularly in field circumstances where thorough hand washing prior to hand sanitiser use is generally not possible.

5.3.4 Equipment

Broad-spectrum bactericidal, virucidal and fungicidal disinfectant spray is readily included in field equipment. Wiping organic matter off all equipment that comes into direct contact with an animal, and then spraying with disinfectant and observing recommended contact times before use in the next animal is considered likely to reduce the risk of cross-contamination of samples, as well as spread of infection.

5.4 Assessing the likelihood of cross-contamination substantially influencing epidemiological data

Post hoc assessment of the potential impact of cross-contamination on research data can quantify potential impact on study results. This allows assessment of the likelihood of results being substantially influenced by cross-contamination. We use the *Giardia* spp. results of our quenda survey to outline the approaches we employed.

In our *Giardia* spp. study, the quenda *Giardia* spp. isolates were characterised by PCR and sequencing at three loci: 18S rRNA, ITS1-5.8s-ITS2 and *gdh* (see Chapter 9.2.4). The results of these analyses indicated that the *Giardia* spp. infections were almost invariably *Giardia peramelis* (Chapters 8 and 9)*.* As the only confirmed host of *G. peramelis* is quenda, quenda were identified and focussed on as the most likely source of cross-contamination regarding the *Giardia* spp. results in this study.

Of the 284 trapped quenda from which we obtained faeces, 99 were found to be infected with *Giardia* spp. Of these, 11 *G. peramelis* positive quenda were trapped subsequent to another *G. peramelis* positive quenda. One of these eleven quenda did not have a single nucleotide polymorphism in the *G. peramelis* isolated from its faecal

sample, although this was identified in the *G. peramelis* detected in the quenda trapped immediately prior. Therefore, this quenda was considered to be truly infected with *G. peramelis,* as it was not plausibly a false result due to cross-contamination from the faeces of the quenda trapped prior. The remaining ten quenda had *Giardia peramelis* infections that were not distinguishable from the infection of the quenda trapped prior, and so remained candidates for possible cross-contamination of the faecal samples.

The burden of infection provides a further indicator of the likelihood of cross-contamination. For example, particularly where biosecurity has been considered in trap management strategies, it is unlikely that if a faecal sample from an animal trapped subsequent to an infected animal was contaminated with *Giardia* sp., rather than intrinsically infected, that it would have a *Giardia* sp. cyst burden that greatly exceeded that of the previously trapped animal. Furthermore, the probability of cross-contamination is likely to increase (or at the very least, stay the same) with a heavier burden of infection in the initially trapped animal. In our study, *Giardia* sp. cyst burdens on immunofluorescence microscopy of the quenda samples were noted and compared. Of the ten quenda identified above as potential candidates for cross-contamination, four had at least a four-fold higher burden of *Giardia* sp. cysts on immunofluorescence microscopy than the *Giardia* sp. positive quenda trapped before them. Though not conclusive, this suggests that these positive samples represented intrinsic infection in that animal, rather than cross-contamination. Additionally, of all quenda (both positive and negative for *Giardia* spp.) that were trapped subsequent to *Giardia* spp. positive quenda, those that were trapped after the three quenda who were shedding the most *Giardia* spp. cysts in their faeces all tested negative.

Finally, we used data analysis to compare the odds of *Giardia* spp. infection in quenda trapped subsequent to a *Giardia* spp. positive quenda, to the odds of *Giardia* spp. infection in quenda not trapped subsequent to a known *Giardia* spp. positive quenda. Using logistic regression, we found that there was no association between a quenda

being trapped subsequent to a *Giardia* spp. positive quenda and the odds of testing positive for *Giardia* spp. (p = 0.97). Quenda trapped subsequent to a *Giardia* spp. positive quenda had 1.03 times the odds of testing positive for *Giardia* spp. infection, compared to those trapped in a trap that had not been previously occupied by an infected quenda (95% CI 0.22 – 4.79).

5.5   Conclusions

Biosecurity in the trapping of wildlife deserves attention at the study design, field data collection, and data analysis stages. It is important for research rigour, and also for human and animal welfare. Depending on laboratory data available, it may be possible to confirm that cross-contamination of samples did not occur. Additionally, a variety of data analyses may either provide support that cross-contamination is not likely to have substantially influenced results, or alert the researcher to potential problems regarding contamination.

5.6   Acknowledgements

5.7 References

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Chapter 6

Parasitic infections of brushtail possums (*Trichosurus vulpecula*) in Perth, Western Australia

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6. Parasitic infections of brushtail possums (*Trichosurus vulpecula*) in Perth, Western Australia

6.1 Introduction

Brushtail possums (*Trichosurus vulpecula*) are a very adaptable marsupial, remaining in many urbanised areas in Australia as well as in bushland. Brushtail possums living in close proximity with humans and domestic animals in urban areas provide a "One Health" combination of concerns from an anthropozoonotic infectious disease perspective: if brushtail possums are acquiring infections from humans and pets, their population health may be adversely impacted, and they may act as a reservoir of such infections to human and domestic animal populations.

Current knowledge of parasitic infections of brushtail possums inhabiting urbanised environments in Australia is limited to studies focussed on the protozoan parasites *Toxoplasma gondii, Neospora caninum* and *Cryptosporidium* spp. in brushtail possums in urban Sydney (Eymann, et al., 2006; Hill, et al., 2008a; Hill, et al., 2008b), and ectoparasites in brushtail possums in a zoological park and surrounds in Sydney (Webster et al., 2014). There is currently limited published information regarding parasites infecting brushtail possums in Western Australia (Viggers and Spratt, 1995; Obendorf, et al., 1998; Adams, 2003; Thompson, et al., 2010; Clarke, 2011), and no studies have investigated parasites of brushtail possums in Perth.

In sampling free-ranging brushtail possums in Perth, Western Australia, this study aimed to:

    1) measure *Toxoplasma gondii* seroprevalence;

    2) identify and estimate the prevalence of gastrointestinal protozoan and helminth parasites;

    3) identify and estimate the prevalence and intensity of ectoparasite infections;

4) compare the odds of endo- and ecto- parasitism in brushtail possums in urbanised environments *vs.* bushland;

5) compare the body condition of adult possums trapped in urbanised environments and bushland; and

6) compare the pouch activity of adult females in urbanised environments and bushland.

6.2   Methods

This cross sectional study targeted free-ranging brushtail possums inhabiting both urbanised environments and bushland within the Statistical Division of Perth. The source population and results were stratified into brushtail possums trapped on bushland sites, brushtail possums trapped on urbanised sites, and brushtail possum carcasses obtained via a wildlife hospital. Trapping sites were classified as bushland if included in the Western Australian Planning Commission's Bush Forever policy (Department of Planning, 2000). Trapping sites were classified as urbanised if they were private properties (including both residential and non-residential sites).

6.2.1   Trapping and sampling brushtail possums on bushland and urbanised sites

As the population distribution of marsupial species in Perth is heterogeneous and poorly described, bushland sites were selected for trapping after consultation with fauna scientists and assessment of many potential trapping sites. Urbanised sites were primarily recruited to the study via a register held by WWF-Australia from the Community Quenda Survey 2012, run in conjunction with the Department of Parks and Wildlife.

Trapping was undertaken between March 2013 and July 2015, totalling 160 nights across 29 bushland sites and 35 urbanised sites (Figure 7.1). Sheffield traps, baited

with universal bait, pieces of apple and truffle oil, were used. Trap placement at sites initially targeted both quenda (*Isoodon obesulus*) and brushtail possums, to encompass a parallel study of parasites in quenda. Due to very low trapping rates of brushtail possums across the first quarter of trapping, subsequent bushland trap site selection and trapping layout tended to prioritise targeting quenda, to focus limited available resources.

Faecal samples were collected from the traps of brushtail possums, and rated as formed (at least one fully formed faecal pellet present) or poorly formed (no distinct faecal pellets present). Faecal samples were temporarily stored in an insulated container for up to six hours, then preserved. For preservation, 2 mL faeces were thoroughly mixed in to 8 mL 10% buffered formalin, and 2 mL faeces were thoroughly mixed in to 8 mL 70% ethanol. Preserved faecal samples were stored at 4°C until analysis.

Trapped brushtail possums were briefly anaesthetised using isoflurane (I.S.O. 1 mL / mL, V.C.A, Australia) vaporised in medical oxygen via a "Stinger" field anaesthetic machine with a Bain circuit (Advanced Anaesthesia Specialists, Sydney). Morphological measurements were taken from each possum. This included testes measurements in males, to classify as subadult (each testis ≤ 1 cm width) or adult (at least one testis > 1 cm width) (this was based on a dichotomy of body weights using this cut off, indicating that this cut off approximated maturity); pouch activity in females, to classify as subadult (non parous pouch) or adult (parous pouch). Assessment of the upper first molar tooth wear was used to grade the age class of the possum from 1 to 7, as per Cowan and White (1989) - a modification of the scale developed by Winter (1980). Adult females were classified as having an active pouch if lactating, or an inactive pouch if not lactating. A thorough ectoparasite screen, including direct examination, coat brush and examination of the handling bag, was undertaken. Ectoparasites were preserved in 70% ethanol / 5% glycerine solution. One millilitre of

blood was taken from the lateral tail vein, and transferred into a minicollect serum tube, inverted five times, left to clot for approximately 30 minutes and then stored in an insulated container for a maximum of four hours. The sample was then centrifuged at 314 G for 10 minutes, and the serum transferred into a storage tube and frozen at -20°C until analysis.

Body condition of the possum was subjectively graded according to a pre-defined scale, via palpation of fat and muscle mass at the scapula spine, temporal fossa and spine. Body condition score ranged from 1 (emaciated) through 2 (underweight), 3 (optimal), 4 (overweight) and 5 (obese). The skin of the possum was checked for gross evidence of pathology. The fur of the body and tail was inspected for alopecia, fur shearing or fur regrowth; if present, it was subjectively categorised as covering 10% or less of the body surface area, or more than 10% of the body surface area.

6.2.2 Sampling brushtail possum carcasses

Brushtail possum carcasses that became available between September 2013 and February 2015 were collected from a Perth wildlife hospital. Where possible, the hospital provided information on the date of death, the location the possum was found, the likely cause of death, and whether or not the possum was treated with anthelmintics or antibiotics prior to death. Likely cause of death of the possums was determined based on information provided by the wildlife hospital and/ or gross findings on post mortem examination.

Twenty two out of 23 possum carcasses were frozen prior to collection, and defrosted at 4°C for 12 - 36 hours prior to necropsy. One possum carcass was stored at 4°C for 48 hrs between death and sampling. Morphological data and ectoparasites were collected as described for trapped possums. The wildlife hospital provided information as to the presence of pouch or back young at the time of death. Faeces were collected

from the large intestine of possums on necropsy, and preserved described for faeces from trapped possums.

6.2.3 Identification of endoparasites in sampled brushtail possums

Serum samples from 32 trapped possums were analysed using the modified agglutination test kit (bioMérieux, France), according to manufacturer's instructions. One possum serum sample was sent for analysis at the Animal Health Laboratory of the Department of Primary Industries, Parks, Water and Environment (Tasmania). Samples were tested at titres of 1:40 and 1:4000, and possums were considered positive for *T. gondii* antibodies if they tested positive at either or both titres.

Formalin-preserved faecal samples were screened for *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts using Merifluor Cryptosporidium / Giardia kit tests (Meridian Bioscience, Inc. USA), according to manufacturer's directions for unconcentrated faecal samples. Slides were examined at 200x magnification and considered positive if at least one cyst of the respective genera was identified by the apple-green fluorescence and appropriate morphology.

Molecular characterisation of *Giardia* spp. was attempted for samples positive for *Giardia* spp. on immunofluorescence microscopy. Faecal samples preserved in 70% ethanol had DNA extracted for amplification at the 18S rRNA, ITS1-5.8S-ITS2 and *gdh* loci, with sequencing of amplified product, using the methods described in Chapter 9.

Gastrointestinal protozoan and helminth infections were identified using zinc sulphate flotation and sodium nitrate faecal flotation methods. Formalin-preserved faecal samples were centrifuged at 850 G for two minutes, with formalin supernatant discarded. The sample was then rehomogenised in distilled water and centrifuged at 850 G for two minutes, with supernatant discarded. The 2 mL of faeces was halved

between two 10 mL centrifuge tubes. One tube was mixed thoroughly with zinc

sulphate solution (SG 1.20) and centrifuged at 850 G for two minutes. A flamed wire

loop was used to transfer the surface material to a slide; a coverslip was added and it

was examined at 200x magnification. The second tube of faeces was thoroughly

mixed with sodium nitrate solution (SG 1.37), centrifuged for two minutes at 850 G, and

then topped up with the sodium nitrate solution to form a meniscus. A coverslip was

placed upon the top of the tube, and left for 10 minutes before being transferred to a

slide. This slide was examined at 200x magnification. A sample was considered

positive if at least one protozoan cyst or helminth egg of the respective morphological

type was identified on either or both flotations.

6.2.4 Identification of ectoparasites on sampled brushtail possums

Ectoparasites were differentiated morphologically. Fleas were mounted directly into a

drop of lactophenol on a microscope slide. Coverslips were added and slides were left

for a minimum of 12 hours to clear (some fleas required up to a week). An Olympus

CH40 compound microscope was used to identify the fleas at magnification of 40 -

400x. All fleas were keyed out using Dunnet and Mardon (1974)**.** Ticks were cleared,

mounted and examined microscopically as described for fleas, though cavity slides

were used for larger or engorged specimens. Ticks were keyed out using Roberts

(1970). The mesostigmatan mite was mounted in Hoyers medium on a microscope

slide, and coverslipped. The mite was examined microscopically and differentiated

using Domrow (1987). Trombiculid mites were initially categorised as such based on

their distinctive macroscopic appearance on the possum sampled. A few mites from

each infected possum were mounted in Hoyers medium on a microscope slide with a

coverslip. Where larval mites were confirmed microscopically, the samples were

categorised as trombiculids.

6.2.5   Mapping and statistical analysis

The locations of sampled possums were mapped using ArcMap 10.3.1 (Esri, 2015). Trapped possums were mapped using the GPS location of the trap in which they were caught. Possums from the wildlife hospital were mapped using the central location of the suburb in which they were located (prior to admission to the wildlife hospital) as a proxy.

Parasite prevalences were calculated with Jeffrey's 95% confidence interval. Prevalences were compared between possums trapped in urbanised environments to those trapped in bushland using the χ^2 test (wildlife hospital possums were excluded from these analyses).

The odds of endo- and ecto- parasitism was then compared in brushtail possums trapped in bushland *vs.* urbanised environments (wildlife hospital possums were excluded from these analyses). Three binary outcomes were assessed: 1) infected with any gastrointestinal protozoan taxa, 2) infected with any gastrointestinal helminth taxa, 3) infected with any ectoparasite taxa. Putative confounders considered were sex and age group (younger possums = tooth wear class 1-2, older possums = tooth wear class 3+). Statistical analyses were undertaken using Stata data analysis software (StataCorp, 2015). Association between the exposure and outcome were assessed using univariable logistic regression. Data were assumed to be independent, due to the small numbers of possums within many putative trapping location clusters (many n = 1). Where univariable associations provided at least weak evidence against the null hypothesis (p ≤ 0.10), multivariable models were built. Each putative confounder was sequentially added to the model, and retained if it changed the odds ratio for the association of interest by ≥ 10%. Odds ratios were calculated with Wald p-values.

Pouch activity between adult females in bushland and urbanised environments, and body condition between possums trapped in bushland and urbanised environments, were compared using χ^2 tests.

6.2.6 Ethics approval and sampling permits

All samples were obtained under Murdoch University Animal Ethics Permit R2530/12, and Department of Parks and Wildlife Regulation 17 (SF009640) and Regulation 4 (CE004287) permits.

6.3 Results

6.3.1 Brushtail possums sampled - descriptive findings

Fifty six brushtail possums were sampled. This included: 15 live possums trapped across nine urbanised sites; 18 live possums across seven bushland sites; and 23 fresh / fresh-frozen possum carcasses obtained from the wildlife hospital (Figure 6.1).
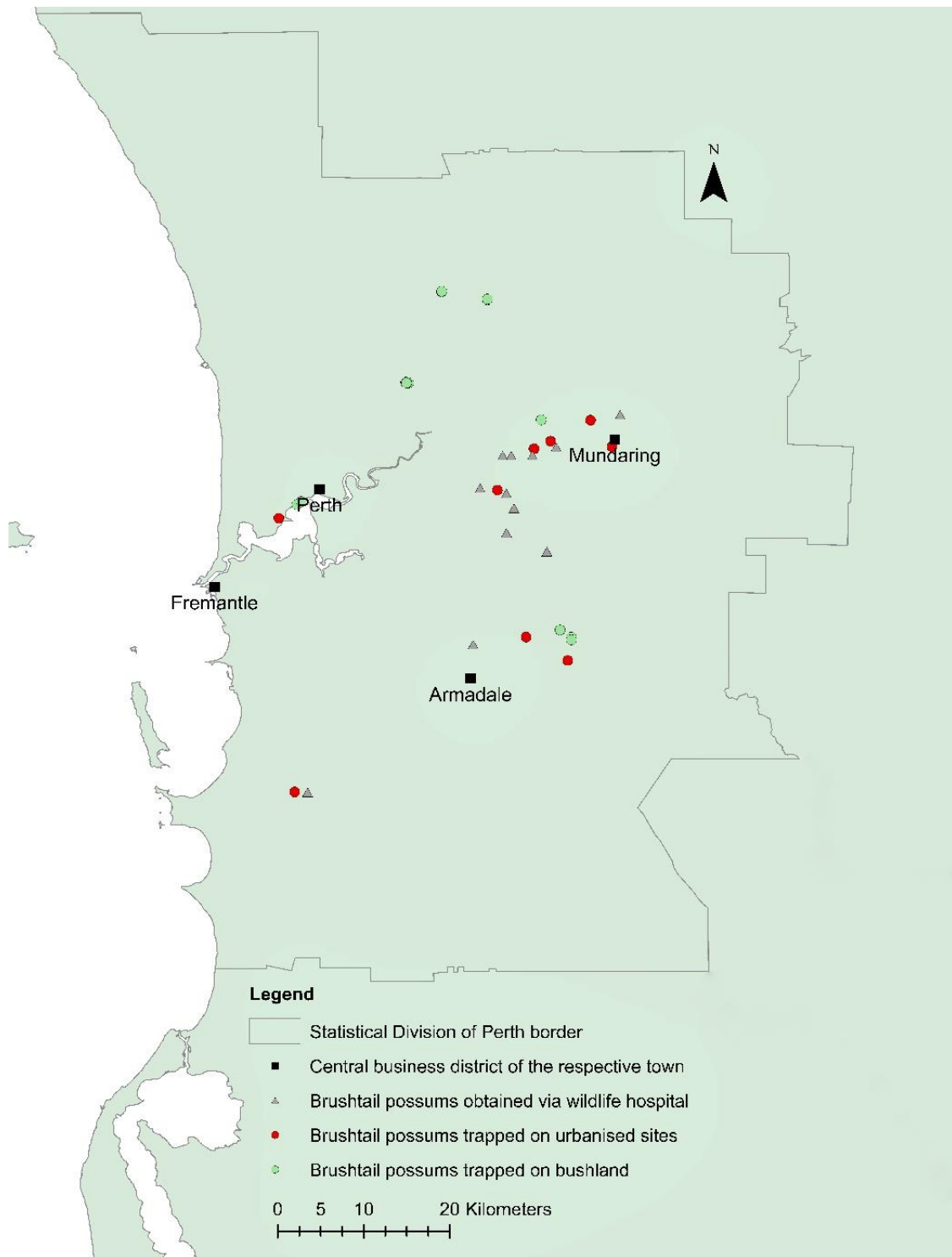
Figure 6.1 Locations of brushtail possums sampled, Perth

Sixteen of the possums obtained from the wildlife hospital were known or judged to have died due to accident or injury (attacked by another animal, electrocution, vehicle strike, ingested poison or chainsaw injury). One possum had been in long term care for neurological symptoms. Six possums did not have clinical history or macroscopic pathology that suggested a likely cause of death.

Five out of 19 of the wildlife hospital possums from which a faecal sample was obtained had been treated with antibiotics prior to death (antibiotic treatment records were not available for the other three possums from which a faecal sample was obtained). Three of these five possums treated with antibiotics had protozoan oocysts in their faeces - two had coccidian oocysts morphologically consistent with *Eimeria* spp., and one had *Entamoeba* sp. cysts. None of 16 wildlife hospital possums from which a faecal sample was obtained were known to have been treated with anthelmintic medication (anthelmintic treatment records were not available from six possums). Of the six possums where anthelmintic treatment records were not available, two had strongyle eggs present in their faeces.

Sampled possums included five subadult males, 21 adult males, two subadult females and 28 adult females (Figure 6.2). The age class distribution of sampled possums is summarised in Figure 6.3 (age class was not able to be assessed in one possum sampled from a wildlife hospital, due to traumatic injuries). Thirteen of the 28 adult female possums had an active pouch.
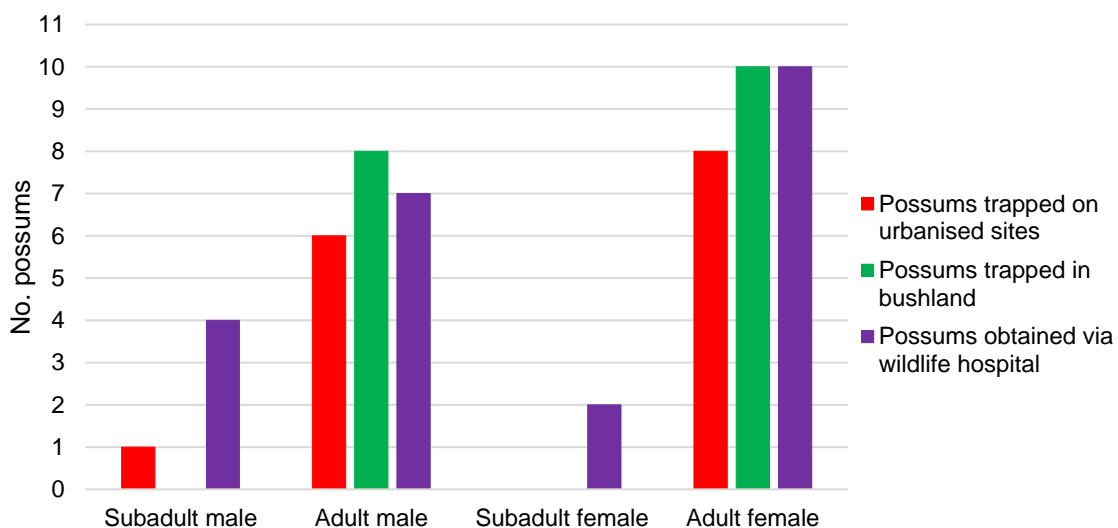
Figure 6.2 Brushtail possums sampled for parasites: sex and maturity, by source population
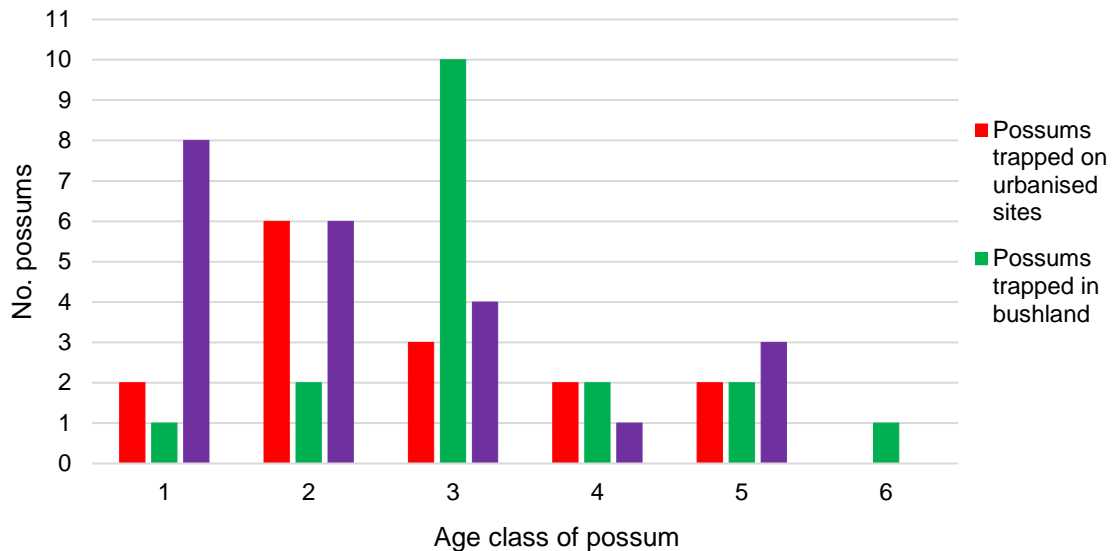
Figure 6.3 Brushtail possums sampled for parasites: age class, by source population
Age classes were estimated using upper left first molar tooth wear, as per Cowan and White (1989) - modified from Winter (1980).

Fifteen of 33 trapped possums were scored as subjective body condition 3 (optimal); 18 possums were scored as subjective body condition 2 (underweight). Eighteen of 23 possums obtained via the wildlife hospital were scored as subjective body condition 3 (optimal); three possums obtained via the wildlife hospital were subjective body condition 1 (emaciated) and two were subjective body condition 4 (overweight). All trapped possums and 22 / 23 possums obtained via the wildlife hospital had no gross skin pathology; one possum had diffuse scale. Thirty of the 33 trapped possums and 22 / 23 wildlife hospital possums had full fur coverage; four possums had fur shearing, fur regrowth and / or alopecia covering less than 10% of the body. All faecal samples obtained from trapped possums were rated as 'formed'.

6.3.2 *Toxoplasma gondii* seroprevalence in Perth brushtail possums

Blood samples were obtained from all trapped possums. No trapped possums tested positive for *Toxoplasma gondii* using the modified agglutination test (Table 6.1).

6.3.3   Gastrointestinal parasites of Perth brushtail possums

Faecal samples were available from 32 trapped possums and 22 possum carcasses. Gastrointestinal parasite findings are summarised in Table 6.1.

Of samples in which unsporulated coccidian oocysts (Family Eimeriidae) were present, the oocysts in 12 samples were morphologically consistent with *Eimeria trichosuri*. The oocysts in the remaining two samples were morphologically consistent with different *Eimeria* spp. The *Entamoeba* spp. cysts observed were not morphologically consistent with *Entamoeba histolytica*. The *Giardia* spp. infection amplified by PCR, with a band at the appropriate size for *Giardia* spp., but sequencing failed so differentiation to species level was not possible.

This data provided no evidence of differing prevalences of gastrointestinal parasites in possums trapped in urbanised environments compared to bushland (Table 6.1).

At the lowest level of taxonomic classification achieved in this study, a single type of parasitic protozoan infection was identified in 21 possums (38.9%); one possum had two concurrent parasitic protozoan infection types identified (1.9%). A single type of parasitic helminth infection was identified in 18 possums (33.3%); one possum had two concurrent helminth infection types (1.9%).

Table 6.1 Endoparasite infection prevalences in Perth brushtail possums (*Trichosurus vulpecula*)

Endoparasites	Trapped possums: No. possums infected / no. possums tested (% infected; 95% CI)			Wildlife hospital: No. possums infected / no. possums tested (% infected; 95% CI)
	Urbanised environments	Bushland	χ² test p-value[1]	
Toxoplasma gondii[2]	0 / 15 (0%; 0 - 20.6%)	0 / 18 (0%; 0 - 17.6%)	-	-
Giardia sp.	1 / 14 (7.1%; 1.7 - 31.9%)	0 / 18 (0%; 0 - 17.6%)	0.25	0 / 20 (0%; 0 - 16.1%)
Cryptosporidium sp.	1 / 14 (7.1%; 1.7 - 31.9%)	0 / 18 (0%; 0 - 17.6%)	0.25	0 / 20 (0%; 0 - 16.1%)
Coccidian oocysts[3]	4 / 14 (28.6%; 11.8 - 55.1%)	4 / 18 (22.2%; 9.1 - 45.6%)	0.68	6 / 22 (27.3%; 13.2 - 48.4%)
Entamoeba spp.[4]	1 / 3 (33.3%; 6.8 - 80.6%)	3 / 11 (27.3%; 9.9 - 57.2%)	0.47	3 / 22 (13.6%; 5.0 - 33.6%)
Strongyle eggs[5]	7 / 14 (50%; 26.6 - 73.4%)	9 / 18 (50%; 28.9 - 71.1%)	1.00	3 / 22 (13.6%; 5.0 - 33.6%)
Unidentified acanthocephalan egg[6]	1 / 14 (7.1%; 1.7 - 31.9%)	0 / 18 (0%; 0 - 17.6%)	0.25	0 / 22 (0%; 0 - 14.8%)

[1] Comparing prevalences in brushtail possums trapped in urbanised environments *vs.* bushland.

[2] Seroprevalence.

[3] Family Eimeriidae, unsporulated; morphology consistent with *Eimeria* spp.

[4] The first 18 trapped possum samples were not screened for *Entamoeba* spp. cysts.

[5] Infraorder Rhabditomorphia.

[6] Phylum Acanthocephala.

6.3.4 Ectoparasites of Perth brushtail possums

Ectoparasite findings are summarised in Table 6.2. Many ticks were larvae or nymphs-some of these were successfully differentiated to a species or genus level, but some could not be differentiated beyond family level.

Ticks (Family Ixodidae) were more prevalent in possums trapped in bushland; this appeared to be particularly attributable to increased prevalence of *Amblyomma* spp. ticks on possums trapped in bushland (Table 6.2).

Ectoparasite intensities of infection were generally low. No more than one flea was obtained from a single possum. Tick burdens ranged from two to seven ticks in infected trapped possums; the one infected possum obtained via the wildlife hospital had a burden of 37 ticks. The single possum on which a *Liponyssoides* sp. mite was identified, had only one mite obtained on screening. Trombiculid mite burdens were not quantified.

Table 6.2 Ectoparasite infection prevalences in Perth brushtail possums (*Trichosurus vulpecula*)

Ectoparasites:		Trapped possums: No. possums infected / total no. possums (% infected; 95% CI)			Wildlife hospital: No. possums infected / total no. possums (% infected; 95% CI)
		Urbanised sites	Bushland sites	X^2 test p-value[1]	
Fleas	*Pygiopsylla tunneyi*	1 / 15 (6.7%; 1.6 - 30.2%)	0 / 18 (0%; 0 - 17.6%)	0.27	0 / 23 (0%; 0 - 14.2%)
	Echidnophaga myrmecobii	1 / 15 (6.7%; 1.6 - 30.2%)	1 / 18 (5.6%; 1.3 - 26.0%)	0.89	0 / 23 (0%; 0 - 14.2%)
	Choristopsylla ochi	0 / 15 (0%; 0 - 20.6%)	2 / 18 (11.1%; 3.4 - 33.1%)	0.18	0 / 23 (0%; 0 - 14.2%)
Ticks	Family Ixodidae[2]	0 / 15 (0%; 0 - 20.6%)	7 / 18 (38.9%; 20.3 - 61.6%)	0.007	1 / 23 (4.3%; 1.0 - 21.1%)
	Ixodes tasmani	0 / 15 (0%; 0 - 20.6%)	1 / 18 (5.6%; 1.3 - 26.0%)	0.35	0 / 23 (0%; 0 - 14.2%)
	Haemaphysalis bremneri[3]	0 / 15 (0%; 0 - 20.6%)	1 / 18 (5.6%; 1.3 - 26.0%)	0.35	0 / 23 (0%; 0 - 14.2%)
	Amblyomma spp.	0 / 15 (0%; 0 - 20.6%)	4 / 18 (22.2%; 9.1 - 45.6%)	0.051	1 / 23 (4.3%; 1.0 - 21.1%)
Mites:	*Liponyssoides* sp.	0 / 15 (0%; 0 - 20.6%)	1 / 18 (5.6%; 1.3 - 26.0%)	0.35	0 / 23 (0%; 0 - 14.2%)
	Trombiculid mites (Suborder Prostigmata)	0 / 15 (0%; 0 - 20.6%)	3 / 18 (16.7%; 6.1 - 39.6%)	0.097	0 / 23 (0%; 0 - 14.2%)

[1] Comparing prevalences in quenda trapped in urbanised environments *vs.* bushland.

[2] On some possums, some or all ticks present could not be differentiated beyond Family Ixodidae (typically larvae and nymph specimens).

[3] The possum infected with *H. bremneri* had another *Haemaphysalis* spp. tick present, which was not able to be differentiated to a species level.

6.3.5 Comparison of endo- and ecto- parasitism between brushtail possums trapped in urbanised environments and bushland

Entamoeba spp., unsporulated coccidian oocysts (morphologically consistent with *Eimeria* spp.) and strongyle eggs were observed in possums trapped in urbanised

environments and in bushland, and in both male and female brushtail possums, across both age groups. Other gastrointestinal parasites were rare and not observed across both age groups and sexes, nor in both urbanised environments and bushland. Ticks and mites were not obtained from possums trapped in urbanised environments. Ticks were obtained from both female and male possums, and across both age groups; fleas and mites were only obtained from possums in the older age group.

On univariable analyses, there was good evidence (p = 0.02) of an inverse association between urbanisation and the odds of a possum being infected with an ectoparasite (p = 0.02). There was no evidence against the null hypothesis of no association between urbanisation and the odds of a possum being infected with a gastrointestinal protozoan or helminth (Table 6.3).

Table 6.3 Univariable logistic regression evaluating associations between urbanisation and infection with any gastrointestinal protozoan, any gastrointestinal helminth, and any ectoparasite, respectively, in Perth brushtail possums (*Trichosurus vulpecula*)

Outcome:		Crude OR[1]	95% CI	p-value
Infected with any gastrointestinal protozoan	Bushland	1		
	Urbanised	2.00	0.48 – 8.40	0.34
Infected with any gastrointestinal helminth	Bushland	1		
	Urbanised	1.00	0.25 – 4.04	1.00
Infected with any ectoparasite	Urbanised	1		
	Bushland	8.13	1.40 – 47.0	0.02

[1] OR= odds ratio.

After controlling for age group and sex, there was weak evidence of an inverse association between urbanisation and the odds of ectoparasitism (adjusted OR = 6.10; 95% CI 0.90 – 41.5; p = 0.064).

6.3.6 Comparison of body condition between brushtail possums trapped in urbanised environments and bushland

Body condition of trapped brushtail possums is summarised in Table 6.4. There was no difference in body condition in possums trapped in urbanised environments *vs.* bushland (p = 0.90).

Table 6.4 Subjective body condition of brushtail possums trapped in urbanised environments and bushland in Perth

	Subjective body condition:		
Trap site:	2 (underweight) No. possums (%)	3 (optimal) No. possums (%)	Total No. possums (%)
Urbanised	8 (53.3%)	7 (46.7%)	15 (100%)
Bushland	10 (55.6%)	8 (44.4%)	18 (100%)

6.3.7 Comparison of pouch activity between adult female brushtail possums trapped in urbanised environments and bushland

Pouch activity of trapped adult female brushtail possums is summarised in Table 6.5. There was no difference in pouch activity between adult female possums trapped in urbanised environments *vs.* bushland (p = 0.14) (Table 6.5)

Table 6.5 Pouch activity of adult female brushtail possums in urbanised environments and bushland in Perth

Trap site	Inactive pouch No. possums (%)	Active pouch No. possums (%)	Total No. possums (%)
Urbanised	2 (25%)	6 (75%)	8 (100%)
Bushland	6 (60%)	4 (40%)	10 (100%)

6.4 Discussion

Generally, endo- and ecto- parasite prevalences, and intensity of ectoparasite infections, were low in brushtail possums in Perth. This contrasts markedly to findings

from quenda (syn. southern brown bandicoots) in the same geographical region (Chapter 7). This may reflect factors such as brushtail possums' primarily arboreal habits, that they do not forage in soil, and relatively lower population densities of brushtail possums in Perth.

The possibility that the home range of the brushtail possums trapped on bushland encompassed urbanised environments cannot be excluded. Generally, possums in bushland were trapped on large reserves, and urbanised environments were not considered likely to comprise a substantial proportion of their home range. Any misclassification to this effect, where comparisons were made between possums trapped in urbanised environments and bushland, would be reflected as an increased risk of Type II error; therefore, where associations were identified they remain valid, though possibly underestimated. By comparing possums trapped in bushland and urbanised environments within the same geographical region (the greater Perth region), potential biases in comparing urban Perth animals to geographically remote bushland (where isolation from urbanisation is guaranteed more reliably) have been avoided. For example, a range of environmental factors, which may influence parasite epidemiology through differences in baseline brushtail possum population density and parasite survival in the environment, and the presence of sympatric native animal species of relevance to the epidemiology of certain parasites that have not been present in greater Perth region for a long period of time for reasons unrelated or additional to urbanisation, may alter parasite epidemiology between the sites for reasons unrelated to urbanisation.

6.4.1 *Toxoplasma gondii* seroprevalence in Perth brushtail possums

Serological results obtained in this study, though limited by the small sample size and non- proportionate sampling, reflect the low *T. gondii* seroprevalences observed in brushtail possums surveyed in other Australian locations (Cook and Pope, 1959;

O'Callaghan and Moore, 1986; Eymann et al., 2006; Hill et al., 2008a; Parameswaran, 2008; Clarke, 2011; Hollings et al., 2013). While this test is a highly specific indicator of *T. gondii* infection in brushtail possums (Chapter 4), the sensitivity has not yet been ascertained and so the potential impact of misclassification on this result, in terms of its representation of infection prevalence, is unknown.

6.4.2 Gastrointestinal parasites of Perth brushtail possums

Our findings suggest that, with the exception of strongyles, gastrointestinal parasite prevalences in Perth brushtail possums are low. However, we cannot exclude bias in these estimates as a result of non-proportionate sampling. Additionally, misclassification may have been an influence: faecal flotations have not been validated for use in identifying gastrointestinal protozoan or helminth infections present in brushtail possums, but based on validation in other species, it is considered likely that the flotations are not 100% sensitive (e.g. Chapter 4; Williamson et al., 1998; Christie et al., 2011; Rinaldi et al., 2011).

Regarding wildlife hospital carcasses, we believe the apparent accidental nature of the majority of the animals' deaths may have avoided substantial bias of parasite prevalence estimates towards that of "sickly" animals. The results suggest that apparent prevalences of protozoan infections are unlikely to have been substantially influenced by the administration of antibiotic medications that may have been capable of treating protozoan infections or reducing protozoan shedding in faeces. Similarly, results suggest that apparent prevalences of gastrointestinal helminth infections are unlikely to have been substantially influenced by the administration of anthelmintic medication. While anthelmintic medication records were not available for six carcasses from which faeces were obtained, the nature of their deaths suggested that they probably arrived at the hospital dead and thus would not have received anthelmintics. The observation of strongyle eggs in the faeces of two of these possums supports this.

Coccidian oocysts identified on microscopy were morphologically consistent with *Eimeria* spp. (typically *Eimeria trichosuri*), however neither sporulation or genetic characterisation were attempted to confirm this. The strongyle egg morphologies were nonspecific. No acanthocephalan worms have been previously identified in brushtail possums - the eggs observed as part of this study may have been a spurious finding, or could plausibly represent a species not yet identified in brushtail possums.

This is the first time that the protozoans *Giardia* sp. and *Cryptosporidium* sp. have been recorded in brushtail possums in Western Australia. *Giardia* spp. was not found in two previous surveys, across which a total of 378 brushtail possums from locations throughout Western Australia (outside of Perth) were sampled (Adams, 2003; Thompson, et al., 2010). *Cryptosporidium* spp. have been previously identified in brushtail possums in Sydney, and findings of that study suggested that *Cryptosporidium* spp. in urban possums are unlikely to be of public health significance (Hill, et. al, 2008b).

6.4.3 Ectoparasites of Perth brushtail possums

Ectoparasite prevalences tended to be low, with a relatively increased prevalence of ticks on possums trapped in bushland. However, observed prevalences may have been biased by non-proportionate sampling, and lack precision due to the small sample sizes. The generally low ectoparasite prevalence and abundance observed in possums obtained via the wildlife hospital may have been biased by administration of ectoparasiticides, manual ectoparasite removal and / or ectoparasites fleeing the carcass after death of the possum.

To our knowledge, this is the first time *Pygiopsylla tunneyi* and *Liponyssoides* sp. have been recorded infecting brushtail possums. *Pygiopsylla tunneyi* has previously been identified infecting quenda, western barred bandicoots (*Perameles bougainville)* and chuditch (*Dasyurus geoffroii*) in Western Australia (Mardon and Dunnet, 1972; Bennett,

et al., 2007; Thomasz, 2014). *Liponyssoides* spp. have been previously identified infecting birds in the Kimberley region of Western Australia and Queensland (Domrow, 1979, 1987). This study has also documented *Haemaphysalis bremneri* infecting brushtail possums in Western Australia for the first time - *H. bremneri* has been previously documented infecting brushtail possums in Queensland, Australia (Roberts, 1963; Heath et al., 1986). These findings may be of importance if future research identifies these ectoparasites as vectors of infections or intermediate hosts of parasites of importance to wildlife health or public health.

6.4.4 Comparison of endo- and ecto- parasitism between brushtail possums trapped in urbanised environments and bushland

The absence of ticks identified on brushtail possums trapped in urbanised environments in Perth suggests that possums may not be important in the epidemiology of tick borne zoonoses in urban areas. Our finding contrasts to that of Webster et al. (2014), who found a variety of ticks in brushtail possums in an urbanised area of Sydney, Australia. This may reflect a variety of environmental and brushtail possum population heterogeneities between the two cities, and as different species of ticks were identified in Sydney study and this one, differences in tick biology may alter the impacts of urbanisation on their epidemiology.

Though the analyses in this study are consistent with substantially increased odds of ectoparasitism in possums trapped in bushland compared to urbanised environments, they provide only weak evidence against the null hypothesis. This may be attributable to Type II error, due to the small sample sizes. Webster et al. (2014) found differing prevalences of some ectoparasite species between brushtail possums in an urbanised environment in Sydney and a bushland location in the Blue Mountains (relatively increased prevalences of *Ixodes trichosuri* and *Echidnophaga myrmecobii* in urbanised environments, and relatively increased prevalence of *Atellana papilio* in bushland). However, these findings may have been biased by geographically-related factors

between the locations, and the comparisons were made using repeated measurements from some individuals which were not accounted for statistically, increasing the risk of Type I error in these findings.

6.4.5 Comparison of body condition between brushtail possums trapped in urbanised environments and bushland, and pouch activity between adult female brushtail possums trapped in urbanised environments and bushland

These comparisons are limited by a lack of power, due to the small sample sizes. However, the body condition findings were very consistent between possums trapped on urbanised environments and bushland. The variable pouch activity findings of adult females, if not a chance finding, may be attributable to seasonal variations in the sampling of brushtail possums in urbanised environments and bushland, given that brushtail possums are seasonal breeders.

6.4.6 Trapping brushtail possums in urbanised environments using ground traps

We had low success trapping urban dwelling brushtail possums using Sheffield traps set on the ground. We failed to trap any possums over two nights on many private properties where property owners reported commonly observing possums about the rooftops and trees. This suggests that future studies of brushtail possums in urbanised Perth may have greater success and efficiency by utilising an alternative mode of capture, such as arboreal traps.

6.5 Conclusions

This study is the first documentation of parasites of brushtail possums in Perth, Australia. Findings include the first recording of the ectoparasites *Pygiopsylla tunneyi* and *Liponyssoides* sp. infecting brushtail possums, and the first recordings of the

ectoparasite *Haemaphysalis bremneri* and endoparasites *Giardia* sp. and *Cryptosporidium* sp. infecting brushtail possums in Western Australia. We found no evidence that Perth brushtail possums are a substantial reservoir of anthropozoonotic parasites. Brushtail possums trapped in urbanised environments in Perth have lower odds of ectoparasitism than those trapped in bushland, and were not found to harbour ectoparasites of known vector-borne disease risk to human health. Larger studies are required to confirm these findings.

6.6 Acknowledgements

6.7　　　References

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Chapter 7

Urbanisation and the quenda: parasites, proportions and parity

This chapter is intended for publication.

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Designed the study: AEH, RCAT

Organised and undertook data collection: AEH

Performed faecal flotation analyses and ectoparasite differentiation: AEH

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7. Urbanisation and the quenda: parasites, proportions and parity

7.1   Introduction

Increasing urbanisation - the proportion of people residing in cities and the land coverage of cities - is a worldwide phenomenon. Globally, 54% of the world's population live in cities, and this is estimated to rise 66% by 2050 (United Nations Department of Economic and Social Affairs, 2014). In Australia, population growth continues (Australian Bureau of Statistics, 2016), and with it urbanisation. Most recent estimates indicate that over two thirds of the Australian population reside in major cities (Australian Bureau of Statistics, 2006).

Urbanisation has important ecological effects. It can cause local extinction of wildlife populations as a result of habitat loss, with a resulting decline in species richness (McKinney, 2002; Garden et al., 2006). Some wildlife species are capable of surviving in urbanised environments, and urbanisation may impact their population health in a variety of other ways. For example, wildlife in urbanised environments are at risk of acquiring infections from humans and domestic pets (Thompson, 2013; Soulsbury and White, 2016). This is of concern from two perspectives: 1) the impact of such infections on wildlife population health; and 2) the potential for wildlife to then become reservoirs of infections that are capable of infecting humans and domestic animals (Daszak et al., 2000; Bradley and Altizer, 2007). Additionally, wildlife in urbanised environments may have access to anthropogenic food sources. An abundance of food sources in urbanised environments may lead to abnormally increased population densities, which may result in increased infection transmission and increased aggression (Daszak et al., 2000, Bradley and Altizer, 2007). Anthropogenic food sources may result in juvenile animals becoming dependent on such food, and may also result in nutritional

imbalances that can lead to metabolic disorders and obesity - though there is not yet sufficient empirical evidence to support this (Orams, 2002).

In Perth, Western Australia, the small marsupials quenda (syn. southern brown bandicoots, *Isoodon obesulus*) have remained in many areas despite urbanisation (Howard et al., 2014). Quenda are terrestrial omnivores, and each will turn over up to approximately four tonnes of soil per year in foraging for their primary foodstuffs of invertebrates, tubers and fungi (Quin, 1988; Valentine et al., 2013). This may put them at particular risk of soil transmitted infections including, in urban areas in particular, infections originating from domestic pet faeces, and human faeces where septic tanks are in use. Quenda will opportunistically scavenge meat (O'Brien et al., 2010), and are known to be offered meat products in urban areas of Perth, sometimes raw, alongside a variety of other supplementary foodstuffs (Chapter 3; Howard et al., 2014). These foodstuffs may act as a source of various infections to quenda, and supplementary feeding may adversely affect population health and dynamics. To date, however, no studies have addressed the question of whether quenda in urban and bushland environments differ in health or infection status.

In this study, we aimed to:

1) identify gastrointestinal and macroscopic ecto- parasites infecting free-ranging quenda in the greater Perth region, and measure *T. gondii* seroprevalence;

2) compare *T. gondii* seroprevalence, gastrointestinal and ecto- parasite prevalences, and the odds of infection with gastrointestinal and ecto-parasites, in quenda trapped in urbanised environments *vs.* bushland;

3) compare ectoparasite intensities of infection in quenda trapped in urbanised environments *vs.* bushland;

4) compare the composition of gastrointestinal and ectoparasite communities of quenda trapped in urbanised environments *vs.* bushland, with consideration of the potential influences of season, maturity and sex on these;

5) compare the morphometrics of adult quenda trapped in urbanised environments *vs.* those trapped in bushland; and

6) compare the reproductive status of adult female quenda trapped in urbanised environments *vs.* those trapped in bushland.

7.2   Methods

This cross-sectional study targeted free-ranging quenda inhabiting both urbanised environments and bushland in the Statistical Division of Perth. As the population distribution of free-ranging quenda in Perth is not comprehensively documented, fauna scientists were consulted and many bushland sites were assessed in selecting those in which trapping was undertaken. 'Bushland' sites were classified as such based on their inclusion in the Western Australian Planning Commission's Bush Forever policy (Department of Planning, 2000). However, quenda trapped within a 200 m radius of urbanised centres of bushland (offices with food outlets and barbecue areas) were defaulted to 'urbanised'. Selected bushland sites were usually relatively large reserves, in consideration of the estimated average home range of 2.85 ha for male quenda in Perth bushland (female quenda tend to have smaller home ranges) (Broughton and Dickman, 1991). In some cases, the bushland reserves were fenced, preventing quenda trapped on these sites having home ranges that encompassed adjacent urbanised areas. In some other cases, barriers such as fenced train lines, main roads, industrial areas that were considered unlikely to provide quenda with usable habitat, or tracts of cleared but not yet developed / inhabited land, were considered likely to provide barriers or buffers to quenda trapped on bushland having a home range incorporating adjacent urbanised areas. Private residential and private non-residential properties were classified as urbanised sites. Urbanised sites were primarily recruited

to the study via a register held by WWF-Australia from the Community Quenda Survey 2012, run in conjunction with the Department of Parks and Wildlife.

7.2.1 Trapping and sampling quenda

Trapping for this project was undertaken between March 2013 and July 2015. Twenty-nine bushland sites and 35 urbanised sites (28 private residential properties and 7 private non-residential properties) were trapped, across a total of 160 nights (Figure 7.1). Trapping occurred primarily in spring and autumn; however, at two sites collaboration with other research projects entailed trapping in winter.
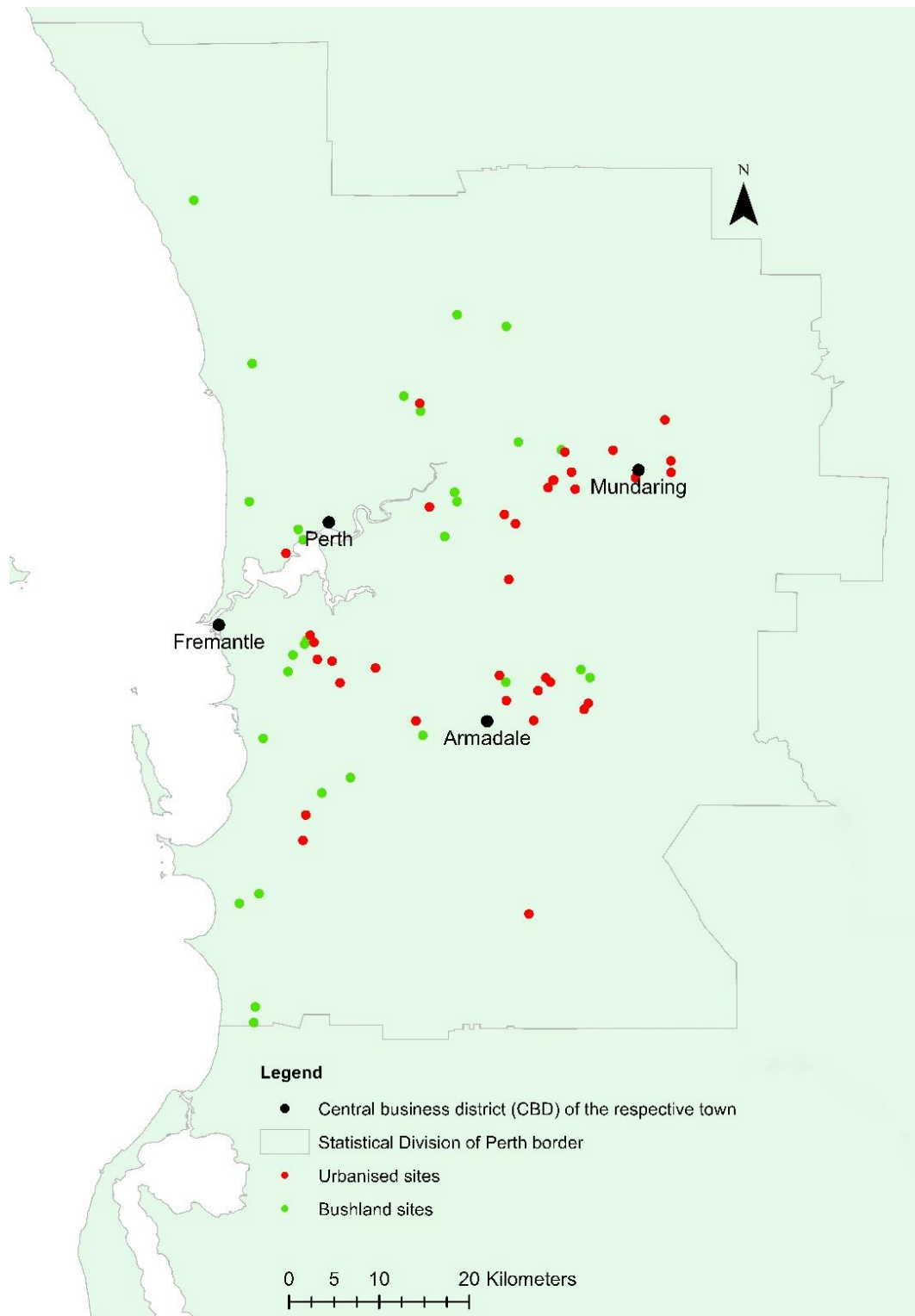
Figure 7.1 Locations of trapping sites for quenda in the greater Perth region.
Sites mapped using the GPS point of the trap of the first quenda trapped, or the GPS point of
the first trap where no animals were trapped.

Quenda were trapped using Sheffield traps baited with universal bait (peanut butter,

oats and sardines), pieces of apple and truffle oil. Traps were covered with hessian

bags lined with heavy plastic. Due to concerns about the safety of trapped animals -

particularly in urbanised sites, but also in bushland accessible to humans - trap layout at sites prioritised safety of the trapped animals. As a result, trap layout was usually not in a rigorous form suitable for population density estimates.

Clean newspaper underlaid each trap, to aid collection of faeces. Faecal samples were stored in an insulated field box for up to 6 hours prior to processing. Processing entailed clearing the sample of contaminants and preservation. Two mL faeces was preserved in 8 mL 10% buffered formalin, and 1 mL faeces was preserved in 8 mL 70% ethanol.

Trapped quenda were weighed, and then a "Stinger" field anaesthetic machine with a Bain circuit (Advanced Anaesthesia Specialists, Sydney) was used to briefly anaesthetise the quenda with isoflurane (I.S.O. 1 mL / mL, V.C.A, Australia) vaporised in medical oxygen. Under light anaesthesia, morphological measurements were taken. These included: 1) head length, measured from the occiput to the tip of the nose, using callipers (measured in cm, to two decimal places); 2) average long pes length - each pes was measured from the base of the heel (behind the calcaneus) to the base of the claw on digit IV, using callipers. Pes length was measured in cm, to two decimal places, and the two pes measurements were averaged; 3) scrotal width, measured using callipers, in cm to two decimal places; and 4) "subjective body condition", graded on qualitative palpation of muscle and fat mass at the temporal fossa, scapula spine and spine - quenda were graded from 1 (emaciated) to 2 (underweight), 3 (optimal), 4 (overweight) and 5 (obese). Pouch development was noted in females, as non-parous (pouch was not developed) or parous (developed pouch). Amongst parous females, pouch activity was noted, regarding lactation and the presence of pouch young.

Male quenda were categorised as adult if their scrotal width was equal to or greater than 2 cm, and subadult if scrotal width was less than 2 cm, based on a markedly increased body weight of quenda with a scrotal width of 2 cm or greater, compared to

those with a scrotal width of less than 2 cm (Dr Tony Friend, Department of Parks and Wildlife, pers. comm.). Female quenda were categorised as adult if they had a parous pouch, and subadult if they had a non-parous pouch.

Net weight was available from quenda without pouch young present. Net weight was used to calculate "objective body condition". This was calculated separately for adult male and adult female quenda, by linear regression using Stata 14 software (StataCorp, 2015). For this calculation, data were restricted to adult quenda who were graded as 3 (optimal) on subjective body condition, to avoid the relatively high levels of obesity in the overall dataset skewing the regression. The average long pes length was regressed against the net weight of the quenda, and the regression equation was used to calculate the expected body weight for each quenda. The residual of the net weight to expected body weight was then used as a measure of "objective body condition".

Each quenda was carefully screened for ectoparasites, including direct inspection of the animal, a coat brush, and inspection of the animal's handling bag both during anaesthesia and after the quenda was released. The aim was to collect all macroscopic ectoparasites present. Ectoparasites were then preserved in 70% ethanol.

One millilitre of blood was collected from the jugular vein using a 25 G needle, transferred into a minicollect serum tube, inverted several times and allowed to clot for approximately 30 minutes. The sample was then stored in an insulated container with an icepack, for a maximum of four hours before processing. Processing involved centrifugation at 314 G for 10 minutes, then transfer of the serum to a storage tube for freezing at -20°C until analysis.

Biosecurity measures were implemented in the trapping and sampling of animals, to minimise the risk of cross-contamination of samples and anthropogenic spread of infection (Chapter 5).

A number of precautions were taken to ensure that quenda at a given site were not resampled. At seven sites (across which 47 quenda were sampled), quenda were microchipped for collaborators, and microchip monitoring ensured no repeat sampling. At the remaining sites, each sampled quenda was marked by a small patch of shaved fur, to ensure identification of previously sampled animals. To minimise the risk of repeat sampling of quenda between sites, site sampling was planned to ensure at least a 1 km radius exclusion around trapped sites for further trapping (sites were closer in one instance, but trapped successively). Additionally, sites that were trapped within relatively close proximity, were typically trapped successively (or simultaneously, on three occasions), so that previously sampled animals would have been readily identifiable.

7.2.2   Identifying parasites that infect free-ranging quenda in Perth

7.2.2.1   Gastrointestinal protozoans and helminths

Formalin-preserved faecal samples were screened for *Giardia* spp. cysts and *Cryptosporidium* spp. oocysts, using a commercial immunofluorescence microscopy kit (Merifluor Cryptosporidium / Giardia, Meridian Bioscience, Inc. USA), according to manufacturer's directions for unconcentrated faecal samples. Slides were examined at 200x magnification. Quenda were considered to be infected with *Giardia* spp. and *Cryptosporidium* spp. if at least one cyst of appropriate fluorescence and the respective morphology was identified.

Other gastrointestinal protozoans and gastrointestinal helminths were tested for using a faecal flotation protocol involving zinc sulphate and sodium nitrate faecal flotations (methodology described in Chapter 4). A sample was considered positive for the respective protozoan or helminth taxon if at least one cyst or egg of that taxon was identified.

7.2.2.2   *Toxoplasma gondii* **seroprevalence**

Quenda serum samples were tested for antibodies to the parasite *Toxoplasma gondii* using a commercial modified agglutination test kit (ToxoScreen DA, bioMérieux, France) at titres of 1:40 and 1:4000, according to manufacturer's instructions. A quenda was considered positive on the modified agglutination test if the test was positive at either or both titres. "Borderline" reactions were defaulted to seronegative.

7.2.2.3   **Ectoparasites**

All ectoparasites were differentiated morphologically, using an Olympus CH40 compound microscope at magnifications of 40 - 400x, and with clearing of specimens undertaken as required. Where clearing was undertaken, fleas were mounted directly into lactophenol on microscope slides, and once clear were differentiated to species level. All fleas were differentiated using Dunnet and Mardon (1974). All flea genera were differentiated to species level, except for *Pygiopsylla*. A subset of 50 *Pygiopsylla* spp. specimens (obtained from 22 hosts across nine bushland and 11 urbanised sites) were differentiated to species level.

Lice were differentiated to Suborder Mallophaga via microscopic examination. Eleven lice (at least one louse per infected host) were mounted directly into lactophenol on microscope slide to clear, and once clear were differentiated to species level using von Kéler (1971).

Fly larvae were differentiated to genus and instar level using spiracle morphology. The posterior end of the larvae were removed and placed directly onto a slide, with a second slide placed on top to squash the spiracles between them. The presence of an additional accessory sclerite between the mouth hooks at the head end was also used to differentiate *Calliphora* sp. from *Lucilia* sp. (Spradbery, 1991; Dunsmore et al., 2002).

Ticks were differentiated to species level microscopically, with clearing undertaken as required. Specimens for clearing were mounted directly into lactophenol on microscope slides, and were differentiated once clear. All ticks were identified using Roberts (1970).

Mites were differentiated to Order Mesostigmata and then into two morphotypes via microscopic examination. Of the first morphotype identified, 25 mites from 11 hosts (hosts obtained from ten sites) were cleared for differentiation to species level. Of the second morphotype, 25 mites from 10 hosts (hosts obtained from nine sites) were cleared for differentiation to species level. Very dark mite specimens were cleared by being placed into a cavity block containing 10% KOH, covered, and left on a low temperature hot plate overnight to de-colour. They were then washed in 70% ethanol and placed directly onto a microscope slide. Paler mite specimens were mounted directly into lactophenol on microscope slides to clear. Once clear, all specimens were differentiated using Domrow (1987).

Trombiculid mites (Suborder Prostigmata) were identified based on their distinctive macroscopic appearance on the quenda, with larval mites confirmed on microscopic examination.

Quenda were considered infected with the respective ectoparasite taxa if at least one specimen was obtained from the quenda and differentiated as such in the laboratory. Intensity of infection data were obtained from all ectoparasite taxa except trombiculid mites.

7.2.3 Mapping

Trapping sites were mapped using the GPS point of the first quenda trapped as the centroid, or the location of the first trap if no quenda were trapped at the site. Quenda

were mapped using the GPS point of the trap in which they were caught. Mapping was undertaken using ArcGIS 10.3.1 (Esri, 2015).

Quenda were also mapped on GoogleEarth (Google, 2015), using the GPS point of the trap in which the animal was caught, which enabled measurement of the closest private property to the trap, where that was of interest.

7.2.4 Data analysis

7.2.4.1 Quenda parasite prevalences, comparison of the odds of parasitism between urbanised environments and bushland, and ectoparasite intensities

Prevalence estimates were stratified into quenda trapped in urbanised environments and bushland, and calculated using Jeffrey's 95% confidence interval. Prevalences were compared using the χ^2 test.

Where there was at least weak evidence of a difference in the prevalence of parasite taxa between quenda trapped in urbanised environments and bushland ($p \leq 0.10$), at the lowest level of parasite taxa possible (i.e. not including Family Ixodidae and *Ixodes* spp.), logistic regression was used to compare the odds of infection between quenda trapped in urbanised environments and bushland. The exceptions were strongyles (Infraorder Rhabditomorphia), as the 100% prevalence in bushland precluded modelling the odds of infection, and findings considered spurious (*Ascaridia* spp. and oxyurids (Order Oxyurida)). Undertaking logistic regression enabled consideration of the potential influence of data correlation and confounding on the prevalence findings.

Data were assessed for spatial autocorrelation by trapping site; if there was not good evidence of correlation (likelihood ratio test of rho=0 $p > 0.05$), independence was assumed and data were analysed using ordinary logistic regression. If there was good evidence of correlation (likelihood ratio test of rho=0 $p \leq 0.05$) data were analysed

using mixed effects logistic regression, with trapping site included in the model as a random effect. The validity of the mixed effects models were checked using quadrature checks, and by comparing each model output to the output of the equivalent generalised estimating equation model (that utilised exchangeable correlation matrix and robust standard errors). If the quadrature check indicated all relative differences were less than or equal to 0.01, and the mixed effects logistic regression output was similar to that of the equivalent generalised estimating equations model, the mixed effects logistic regression model was considered valid and reported in results. If any relative differences in the quadrature check output exceeded 0.01, and / or the mixed effects logistic regression output was markedly different to that of the equivalent generalised estimating equations model, the mixed effect logistic model was considered unreliable and the generalised estimating equations model was reported in the results. Results were reported with Wald p-values.

Where univariable analysis provided at least weak evidence against the null hypothesis of no difference in the odds of a quenda being infected with the respective parasite taxon in urbanised environments compared to bushland ($p \leq 0.10$), confounding was assessed for using multivariable analysis. Confounders considered were: age of the animal (subadult *vs.* adult); sex of the animal; and the season in which the animal was trapped and sampled. Confounders were sequentially added to the model, and retained if their inclusion adjusted the odds ratio for the association by $\geq 10\%$. In the cases of ordinary logistic regression, interaction between confounders and the associations of interest were assessed using the likelihood ratio test. In the cases of mixed effects logistic regression and generalised estimating equations, interaction was tested for using the Wald test. The interaction term was retained in the model if $p \leq 0.05$, and omitted if not.

Ectoparasite taxa intensities were compared between quenda trapped in bushland and urbanised environments, where there were at least five infected hosts in each stratum.

Comparisons of the median intensity of infection were undertaken using the Wilcoxon rank sum test, using Stata 14 software (StataCorp, 2015). Where there was at least weak evidence against the null hypothesis (p ≤ 0.10), findings were further investigated using linear regression, to investigate the potential influence of data correlation and confounding on intensity results. Intensity data were log transformed, then assessed for spatial autocorrelation by trapping site; if there was not good evidence of correlation (likelihood ratio test p > 0.05), independence was assumed and data were analysed using standard linear regression. If there was evidence of correlation (likelihood ratio test of rho=0 p ≤ 0.05) data were analysed using generalised estimating equations models with exchangeable correlation matrices and robust standard errors, as preliminary analyses indicated that the equivalent mixed effects linear regression models were unreliable in all intensity analyses. Results were reported with Wald p-values. In the multivariable analyses, confounding by age, sex and season of trapping and sampling were assessed as per the logistic regression models, as was interaction between putative confounders and the association of interest. However, testing for interaction was omitted from the *I. australiensis* intensity of infection model, as the low number of infected animals across the strata in autumn precluded this.

7.2.4.2 Quenda parasite communities

The null hypotheses of no difference in parasite infracommunity composition in quenda trapped in bushland compared to urbanised environments, across seasons, between sexes and between subadults and adults, were tested by analysis of similarities (ANOSIM) using the software package Primer v5.2.4 (Clarke and Gorley, 2001). Gastrointestinal protozoan and helminth endoparasite infracommunity (binary taxa data: presence/absence of infection) and ectoparasite infracommunity (binary taxa data: presence/absence of infection) were assessed separately as dependent variables. Analyses only considered quenda where at least one taxon of the respective type (endoparasite or ectoparasite) was identified infecting the animal. *Entamoeba* spp. was excluded from the endoparasite analyses, due to missing data. Taxa considered in

the ectoparasite analyses were those at the lowest level of differentiation possible (*A. chera, Pygiopsylla* spp., *S. dasyuri, B. bettongia, Lucilia* spp., *Calliphora* spp., *I. fecialis, I. australiensis, Amblyomma* spp., *H. humerosa*, mesostigmatan mite morphotype 1, mesostigmatan mite morphotype 2 and trombiculid mites).

Similarities in parasite infracommunity composition among all sampled quenda were estimated using the Bray-Curtis coefficient. A permutation procedure applied to the similarity matrix (one-way ANOSIM) was used to test differences in infracommunity for each independent variable (bushland *vs* urbanised environment, season, sex and maturity). Variables in which there was at least weak evidence against the null hypothesis of no parasite infracommunity differences between strata ($p \leq 0.10$), were subsequently tested using two-way ANOSIM. The parasite taxa contribution to the observed dissimilarity between quenda trapped in urbanised environments *vs.* bushland was then estimated using the SIMPER function in Primer v5.2.4 (Clarke and Gorley, 2001). The list of percentage contribution by taxa was cut off after the cumulative percentage community difference had been explained by at least 90%.

7.2.4.3　　　　　Quenda morphometrics

Morphometrics were compared between adult quenda trapped in urbanised environments and bushland, with morphometrics modelled separately for each sex. Comparisons of head length, average long pes length, scrotal width, net weight and objective body condition were undertaken by linear regression, using Stata 14 software (StataCorp, 2015). Linear regression was used in order to obtain an estimate of the average change in morphometrics in quenda trapped in urbanised environments compared to bushland via the regression coefficient, in addition to a hypothesis test of the putative association. Data were assessed for spatial autocorrelation by trapping site; if there was not good evidence of correlation (likelihood ratio test of rho=0 $p >$ 0.05), independence was assumed and data were analysed using standard linear regression. If there was good evidence of correlation (likelihood ratio test of rho=0 $p \leq$

0.05) data were analysed using mixed effects logistic regression, with trapping site included in the model as a random effect. Results were reported with Wald p-values.

Where there was at least weak evidence against the null hypothesis on univariable analysis, the association was further investigated using multivariable analysis. Season, gastrointestinal tract parasite taxa richness, tick (Family Ixodidae) intensity of infection, flea (Order Siphonaptera) intensity of infection and mesostigmatan mite (Order Mesostigmata) intensity of infection were considered as putative confounders of the association between urbanisation and objective body condition in adult male quenda. The tick, flea and mite intensities of infection were considered as ordered categorical variables, based on the number of specimens obtained from the individual (nil = 0 specimens; low = 1 - 9 specimens, high ≥ 10 specimens). Season and gastrointestinal tract parasite taxa richness were considered as putative confounders of the associations between urbanisation and net weight in adult male and adult female quenda (intensities of ectoparasite infections were not considered in these associations, as they were considered to be on the causal pathway). Season was considered as a putative confounder of the association between urbanisation and head length in adult male and adult female quenda, due to potential seasonal differences in cohorts of younger adults between the populations (see section 7.3.4). Putative confounders were sequentially added to the regression model, and retained if their inclusion changed the regression coefficient for the association of interest by ≥ 10%. Interaction between the association of interest and each retained putative confounder was tested for using the likelihood ratio test for interaction, with the interaction term retained in the model if p ≤ 0.05. To ensure model validity, normal distribution of the model residuals was confirmed, and where mixed effects linear regression was undertaken results were compared to that of the equivalent generalised estimating equations model (as described in 7.2.4.1). Results were reported with Wald p-values.

Subjective body condition was compared between adult quenda trapped on bushland and urbanised environments, separately by sex, by χ^2 tests, using Stata 14 software (StataCorp, 2015).

7.2.4.4   Reproductive status of adult female quenda

Adult female quenda were considered to have an active pouch if lactating, or an inactive pouch if not lactating. Proportions of these were calculated with Jeffrey's 95% confidence interval. The odds of an adult female quenda having an active pouch were compared between quenda trapped in urbanised environments and bushland using mixed effects logistic regression, with trapping site included as a random effect (as per methods in 7.2.4.3: likelihood ratio test of rho=0 p < 0.05). Season, gastrointestinal tract parasite taxa richness, tick intensity of infection, flea intensity of infection and mesostigmatan mite intensity of infection were assessed as putative confounders (as per morphometrics, section 7.2.4.3) and sequentially added to the model and retained if the odds ratio for the association of interest changed by ≥ 10%. Interaction between the association and season was tested for using the likelihood ratio test for interaction, with the interaction term retained in the model if p ≤ 0.05. The validity of the mixed effects logistic regression model was checked as described in 7.2.4.1. Results were reported with Wald p-values.

7.3   Results

Two hundred and eighty-seven quenda were trapped and sampled: 161 quenda were trapped across 22 bushland sites, and 126 quenda were trapped across 29 urbanised sites (Figure 7.2). Of the 287 quenda, 11 were subadult females, 117 were adult females, 17 were subadult males and 142 were adult males (Figure 7.3). An additional two subadult quenda were trapped on bushland sites, but were excluded from sampling as their particularly small body sizes were judged to make them inappropriate candidates for anaesthesia. Quenda were trapped in three bushland sites with

urbanised centres - at two of these sites, quenda were only trapped at the urbanised centres next to buildings and were therefore classified as urbanised. At the remaining site, quenda were only trapped in bushland (and managing staff reported no sightings of quenda in the urbanised centre at or before the time of trapping).
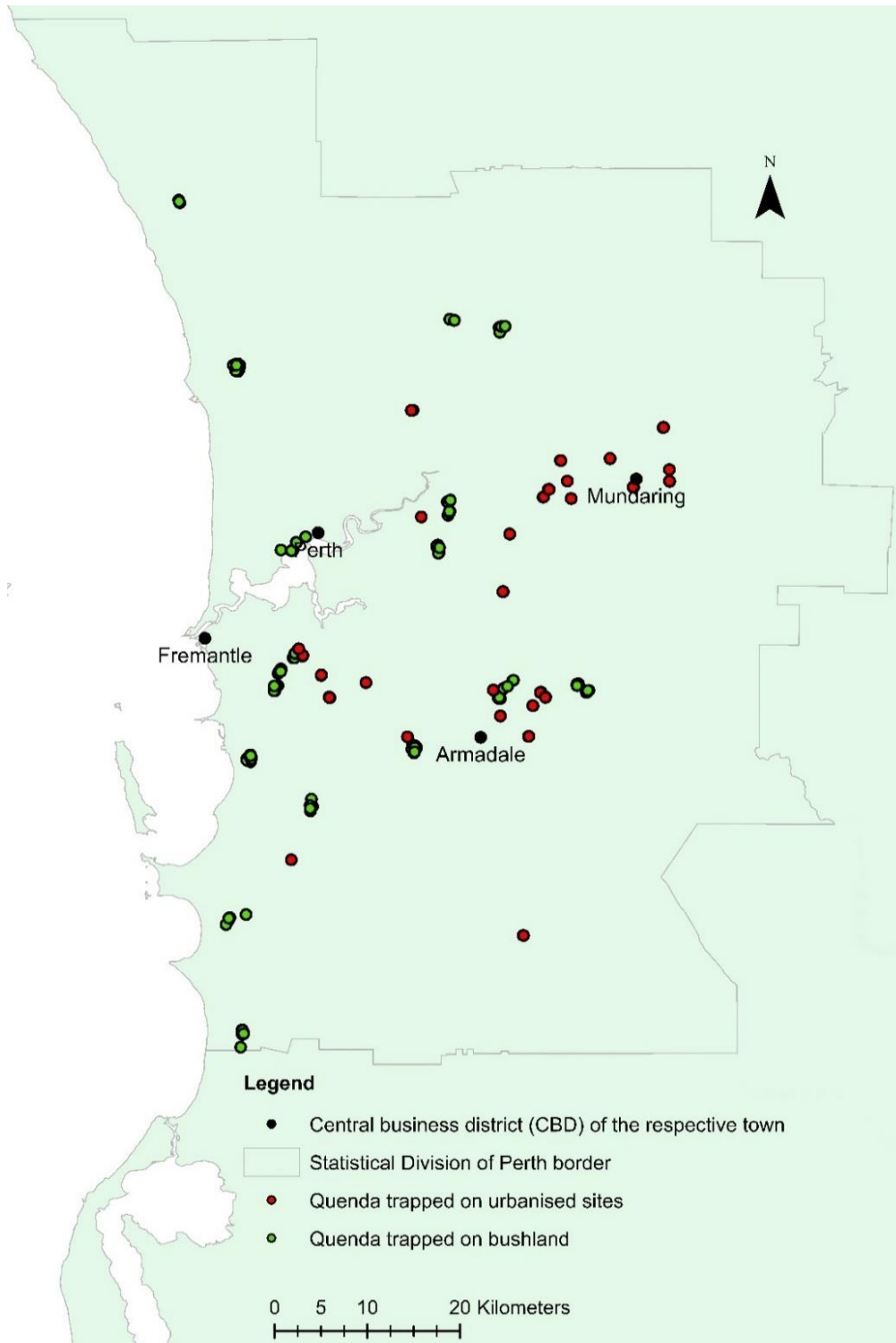
Figure 7.2 Quenda trapped in the greater Perth region
Quenda were mapped using the GPS location of the trap in which they were caught.
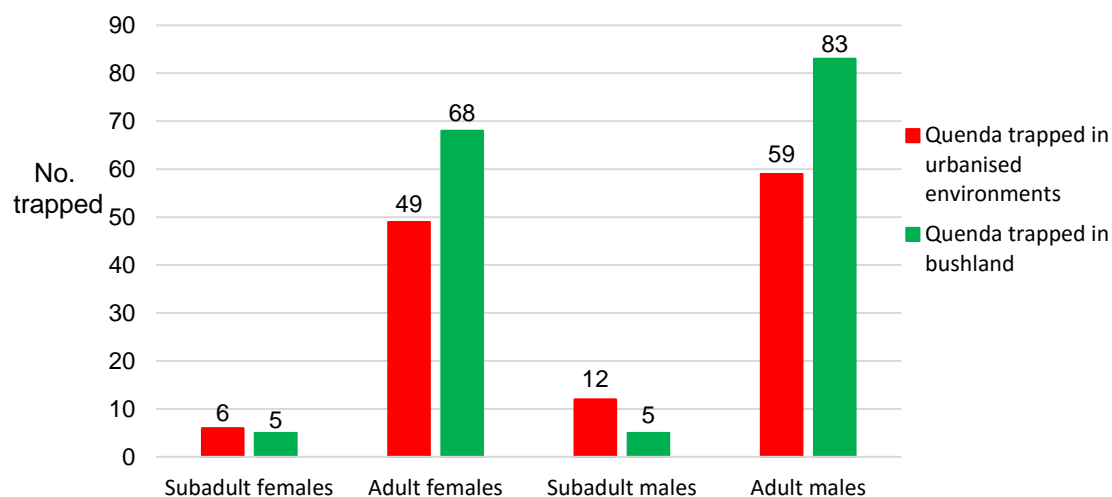
Figure 7.3 Quenda trapped in urbanised environments and bushland in Perth, by sex and maturity

7.3.1　Parasites infecting free-ranging quenda in Perth, and comparisons of parasite prevalences and intensities of infection between quenda trapped in urbanised environments and bushland

7.3.1.1　Gastrointestinal protozoans and helminths

Faecal samples were obtained from 284 / 287 (99.0%) trapped quenda. On faecal flotation analyses, protozoan taxa identified included *Giardia* spp., *Cryptosporidium* spp., *Entamoeba* spp. and coccidian oocysts (Family Eimeriidae). On immunofluorescence microscopy, the *Cryptosporidium* spp. rarely looked typical - the oocysts often had an uncharacteristically thin wall, and were smaller than typical *Cryptosporidium* spp. oocysts (e.g. *C. parvum)*, though a suture line was sometimes visible. The *Entamoeba* spp. infections were not morphologically consistent with *Entamoeba histolytica.*

Giardia spp. and *Cryptosporidium* spp. were more prevalent in quenda trapped in urbanised environments than in bushland (Table 7.1). Correspondingly, quenda trapped in urbanised environments had increased odds of infection with *Giardia* spp. and *Cryptosporidium* spp. infections on univariable logistic regression (Table 7.2). The

increased odds of *Giardia* spp. infection remained after controlling for season of sampling the animal (Table 7.3). However, after controlling for age and season of sampling the animal, there was no evidence of increased odds of *Cryptosporidium* spp. infections in quenda trapped in urbanised environments (Table 7.3).

Nematode taxa identified included strongyles (Infraorder Rhabditomorphia), *Labiobulura* spp., *Linstowinema* spp., *Physaloptera* spp., strongyloids (Family Strongyloididae) and *Trichuris* spp. *Potorolepis* spp. was the only cestode taxon detected.

Linstowinema spp., *Physaloptera* spp. and *Trichuris* spp. were more prevalent in quenda trapped in urbanised areas (Table 7.1). Whilst this was reflected in the increased odds of *Linstowinema* spp. and *Physaloptera* spp. infection on univariable logistic regression (Table 7.2), with no evidence of confounding by age, sex or season of sampling the animal (Table 7.3), there was only weak evidence against the null hypothesis (p = 0.083) of increased odds of *Trichuris* spp. infection via the generalised estimating equations model, with no evidence of confounding by age, sex or season of trapping and sampling the animal (Tables 7.2 and 7.3). Conversely strongyles and strongyloids were more prevalent in quenda trapped in bushland (Table 7.1), however after controlling for the season of sampling the animal there was insufficient evidence of decreased odds of infection with strongyloids in quenda trapped in urbanised environments compared to bushland (Table 7.3). *Potorolepis* was more prevalent in quenda trapped in bushland (Table 7.1), however there was insufficient evidence of differing odds of infection in quenda trapped in urbanised environments compared to bushland on mixed effects logistic regression (Table 7.2).

Unidentified oxyurid eggs (Order Oxyurida) were also identified in the faeces of quenda trapped in both bushland and urbanised environments, whilst *Ascaridia* spp. eggs were also identified quenda trapped in urbanised environments (Table 7.1).

7.3.1.2 *Toxoplasma gondii* seroprevalence

Blood samples were obtained from 158 / 287 (55.1%) quenda. Antibodies to *T. gondii* were detected in five adult quenda- two trapped in urbanised environments and three trapped in bushland (Table 7.1). *Toxoplasma gondii* antibodies were not detected in subadult quenda.

Table 7.1 Prevalences of gastrointestinal protozoan and helminth parasites, and *Toxoplasma gondii* seroprevalence, in quenda trapped in urbanised environments and bushland in Perth

Parasite	Bushland % infected (95% CI) *No. infected / no. tested*	Urbanised environment % infected (95% CI) *No. infected / no. tested*	χ^2 test p-value
Protozoans:			
Giardia spp.	29.2% (22.7 - 36.7%) *47 / 161*	42.3% (33.9 - 51.1%) *52 / 123*	0.022
Cryptosporidium spp.	13.0% (8.7 - 19.1%) *21 / 161*	21.1% (14.9 - 29.2%) *26 / 123*	0.069
Entamoeba spp.[1]	20.7% (14.3 - 29.0%) *24 / 116*	25.6% (14.6 - 41.2%) *10 / 39*	0.52
Coccidian oocysts[2] (Family Eimeriidae)	93.2% (88.2 - 96.1%) *150 / 161*	96.7% (91.9 - 98.7%) *119 / 123*	0.18
Helminths:			
Strongyles (Infraorder Rhabditomorphia)	100% (97.7 - 100%) *161 / 161*	93.5% (87.7 - 96.6%) *115 / 123*	0.001
Labiobulura spp.	83.9% (77.4 - 88.7%) *135 / 161*	84.6% (77.1 - 89.9%) *104 / 123*	0.87
Linstowinema spp.	43.5% (36.1 - 51.2%) *70 / 161*	87.0% (79.9 - 91.8%) *107 / 123*	< 0.001
Physaloptera spp.	1.9% (0.7 - 5.3%) *3 / 161*	9.8% (5.7 - 16.3%) *12 / 123*	0.003
Strongyloids (Family Strongyloididae)	19.3% (13.9 - 26.0%) *31 / 161*	8.9% (5.1 - 15.3%) *11 / 123*	0.015
Trichuris spp.	36.0% (29.0 - 43.7%) *58 / 161*	53.7% (44.9 - 62.2%) *66 / 123*	0.003
Potorolepis spp.	16.2% (11.3 - 22.6%) *26 / 161*	8.1% (4.5 - 14.3%) *10 / 123*	0.044
Spurious helminth eggs:			
Unidentified oxyurids (Order Oxyurida)	20.5% (15.0 - 27.4%) *33 / 161*	4.9% (2.3 - 10.2%) *6 / 123*	< 0.001
Ascaridia spp.	0% (0 - 2.3%) *0 / 161*	4.1% (1.8 - 9.2%) *5 / 123*	0.01
Toxoplasma gondii:			
Seroprevalence	2.9% (1.0 - 8.1%) *3 / 104[3]*	3.7% (1.1 - 12.5%) *2 / 54*	0.74

[1] *Entamoeba* spp. prevalence estimates could not include 129 quenda, due to observer error.

[2] Sporulation of coccidian oocysts was attempted on faecal samples from 71 quenda - all sporulated oocysts were *Eimeria* spp. (Chapter 10)

[3] An additional one bushland quenda was 'borderline'.

Table 7.2 Univariable logistic regression, investigating the association between the respective parasite taxon and urbanisation in Perth quenda

Parasite taxon	Logistic regression type	Independent variable	Crude OR	95% CI	p-value
Giardia spp.	Ordinary	Bushland	1		
		Urbanised environment	1.78	1.08 – 2.91	0.022
Cryptosporidium spp.	Ordinary	Bushland	1		
		Urbanised environment	1.79	0.95 – 3.36	0.071
Linstowinema spp.	Generalised estimating equations	Bushland	1		
		Urbanised environment	8.63	3.73 – 20.0	< 0.001
Physaloptera spp.	Ordinary	Bushland	1		
		Urbanised environment	5.69	1.57 – 20.6	0.008
Strongyloids	Mixed effects	Bushland	1		
		Urbanised environment	0.40	0.16 – 1.00	0.052
Trichuris spp.	Generalised estimating equations	Bushland	1		
		Urbanised environment	1.95	0.92 – 4.16	0.083
Potorolepis spp.	Mixed effects	Bushland	1		
		Urbanised environment	0.45	0.13 – 1.54	0.20

Table 7.3 Multivariable logistic regression, investigating the association between the respective parasite taxon and urbanisation in Perth quenda

Age of the quenda, sex and season of trapping and sampling were considered as potential confounders

Parasite taxon	Logistic regression type	Independent variable	Adjusted OR	95% CI
Giardia spp.	Ordinary	Bushland	1	
		Urbanised environment	1.98	1.18 – 3.32
Cryptosporidium spp.	Ordinary	Bushland	1	
		Urbanised environment	1.79	0.92 – 3.48
Linstowinema spp.	Generalised estimating equations	Bushland	1	
		Urbanised environment	8.63[1]	3.73 – 20.0[1]
Physaloptera spp.	Ordinary	Bushland	1	
		Urbanised environment	5.69[1]	1.57 – 20.6[1]
Strongyloids	Mixed effects	Bushland	1	
		Urbanised environment	0.45	0.18 – 1.10
Trichuris spp.	Generalised estimating equations	Bushland	1	
		Urbanised environment	1.95[1]	0.92 – 4.16[1]

[1] *Linstowinema* spp., *Physaloptera* spp., *Trichuris* spp. - as per crude OR; no evidence of confounding by age, sex and / or season.

7.3.1.3 Ectoparasites

Ectoparasite results were obtained from all 287 quenda. Two thousand, six hundred and forty one fleas (Order Siphonaptera) were obtained from 177 infected quenda. This included 1679 *Acedestia chera* from 107 quenda, 788 *Pygiopsylla* spp. from 146 quenda, 117 *Stephanocirus dasyuri* from 39 quenda, 27 *Echidnophaga gallinacea* from nine quenda and 27 *Echidnophaga myrmecobii* from five quenda (an additional *Echidnophaga* sp. flea and two fleas (Order Siphonaptera) were not able to be more precisely differentiated, due to specimen damage). The 50 *Pygiopsylla* sp. fleas differentiated to species level were all *Pygiopsylla tunneyi*. *Stephanocircus dasyuri* was only found on quenda trapped within the Darling Scarp (hills) area of Perth.

There was at least weak evidence (p ≤ 0.10) of increased prevalence of *Pygiopsylla* spp., *S. dasyuri*, *E. gallinacea* and *E. myrmecobii* infections in quenda trapped in urbanised environments (Table 7.4). However, on logistic regression there was only weak evidence of an association between urbanisation and *Pygiopsylla* spp. infection (p = 0.075) and insufficient evidence of an association between urbanisation and either *S. dasyuri*, *E. gallinacea* or *E. myrmecobii* infection (p > 0.10) (Tables 7.5 and 7.6).

Intensity of *A. chera, Pygiopsylla* spp. and *S. dasyuri* infection are described in Figure 7.4. Of the nine quenda infected with *E. gallinacea,* intensity of infection ranged from one to 10 fleas. The two infected quenda trapped in bushland were trapped in locations within 800m of the nearest private property building, and intensities of infection were one and four fleas, respectively. Of the five quenda infected with *E. myrmecobii*, intensity of infection ranged from one to 14. One *E. myrmecobii* flea was obtained from the single infected quenda trapped in bushland. Median intensity of *Pygiopsylla* spp. infection was higher in quenda in urbanised environments, compared to quenda in bushland (Table 7.7); on linear regression, there remained evidence of increased intensity of *Pygiopsylla* spp. infection in quenda trapped in urbanised environments (Table 7.8), including after controlling for the season in which the animal was sampled

(Table 7.9). There was no difference in median intensities of *A. chera* or *S. dasyuri* infections in quenda trapped in urbanised environments *vs* bushland (Table 7.7).

One hundred and six biting lice (Suborder Mallophaga) were obtained from three infected quenda trapped in bushland (Table 7.4). Each quenda was confirmed to be infected with *Boopia bettongia*; no other lice taxa were identified. Biting lice intensities were 1, 18 and 87. Larvae of the primary strike fly genera *Lucilia* sp. (2 second instar larvae) and *Calliphora* sp. (10 third instar larvae) were obtained from a subcutaneous lesion in one subadult male quenda trapped on an urbanised site (Table 7.4).

One thousand, three hundred and eight ticks (Family Ixodidae) were obtained from 113 infected quenda. This included 354 *Ixodes fecialis* from 58 quenda, 677 *Ixodes australiensis* from 20 quenda, 33 *Haemaphysalis humerosa* from 10 quenda and 128 *Amblyomma* spp. from 48 quenda (an additional 26 *Ixodes* spp. and 90 ticks (Family Ixodidae) could not be differentiated more precisely, due to damage to specimens during the collection process) (Table 7.4). *Ixodes fecialis and I. australiensis* specimens comprised larvae, nymphs and adults. All *Amblyomma* spp. specimens were larvae or nymphs. All *Haemaphysalis humerosa* specimens were adults. *Haemaphysalis humerosa* was concentrated in one bushland site – 9 / 10 infected quenda were from that location.

Ixodes fecialis was more prevalent in quenda in urbanised environments (Table 7.4), and there was evidence of an association between *I. fecialis* and urbanisation on logistic regression (Table 7.5). After controlling for sex, quenda trapped on urbanised environments were found to have 6.34 times the odds of *I. fecialis* infection than those trapped in bushland (95% CI 1.58 – 25.5) (Table 7.6). Conversely, *H. humerosa* was more prevalent in quenda trapped in bushland (Table 7.4); however, on univariable logistic regression there was insufficient evidence of differing odds of *H. humerosa*

infection in quenda trapped in urbanised environments compared to bushland (Table 7.5).

Tick intensities, except for *H. humerosa,* are summarised in Figure 7.5. *Haemaphysalis humerosa* intensities varied from 1 - 9 ticks in bushland; the single infected quenda trapped in an urbanised environment had two ticks. There was at least weak evidence ($p \leq 0.10$) of increased median intensities of *Ixodes* spp. infections in quenda trapped in bushland, and this was reflected in univariable linear regression (Table 7.8). However, after controlling for season the data only supported higher *I. australiensis* intensity of infection in quenda trapped in bushland - there was insufficient evidence of increased intensity of infection of *I. fecialis* in bushland (Table 7.9). There was no evidence of a difference in median intensity of *Amblyomma* spp. infections (Table 7.5).

Three thousand, nine hundred and fifty eight mesostigmatan mites (Order Mesostigmata) were obtained from 170 infected quenda (Table 7.4). These formed two distinct morphotypes. The first morphotype (3860 mites from 166 quenda) comprised mites of varying sizes with ovoid idiosoma. Of the 25 mites differentiated to a species level, 22 were *Haemolaelaps marsupialis*, two were *Gymnolaelaps annectans* and one was *Mesolaelaps australiensis*. The second morphotype (98 mites from 33 quenda) comprised relatively large specimens with distinctly circular idiosoma. Of the second morphotype, all 25 mites differentiated to a species level were *Mesolaelaps antipodianus*.

There was no difference in prevalence of the first mesostigmatan mite morphotype between bushland and urban environments, but the second morphotype was more prevalent in bushland (Table 7.4). However, on logistic regression there was insufficient evidence of an association between urbanisation and infection with the second mesostigmatan morphotype (Table 7.5).

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Intensities of mesostigmatan mite infections are shown in Figure 7.6. The median intensity of infection with the first morphotype was higher in quenda trapped in urbanised environments; this remained the case on linear regression (Table 7.8), and after controlling for the season in which the quenda was trapped and sampled (Table 7.9). There was no difference in the median intensity of infection with the second morphotype between bushland and urbanised environments (Table 7.7).

Trombiculid mites (Suborder Prostigmata) were obtained from 44 quenda. They were more prevalent on quenda trapped in bushland (Table 7.4), however the univariable regression did not provide evidence of differing odds of infection between animals trapped in bushland and urbanised environments (Table 7.5).

Table 7.4 Prevalences of ectoparasites in quenda trapped in urbanised environments and bushland in Perth

Parasite	Bushland % infected (95% CI) *No. infected / no. tested*	Urbanised environment % infected (95% CI) *No. infected / no. tested*	χ^2 test p-value[1]
Fleas:			
Order Siphonaptera[2]	59.6% (51.9 - 66.9%) *96 / 161*	64.3% (55.6 - 72.1%) *81 / 126*	0.42
Acedestia chera	36.6% (29.6 - 44.3%) *59 / 161*	38.1% (30.1 - 46.8%) *48 / 126*	0.80
Pygiopsylla spp.[3]	42.9% (35.5 - 50.6%) *69 / 161*	61.1% (52.4 - 69.2%) *77 / 126*	0.002
Stephanocircus dasyuri	9.3% (5.8 - 14.8%) *15 / 161*	19.0% (13.2 - 26.8%) *24 / 126*	0.017
Echidnophaga gallinacea	1.2% (0.4 - 4.4%) *2 / 161*	5.6% (2.8 - 11.0%) *7 / 126*	0.037
E. myrmecobii	0.6% (0.1 - 3.4%) *1 / 161*	3.2% (1.3 - 7.9%) *4 / 126*	0.10
Lice:			
Suborder Mallophaga	1.9% (0.7 - 5.3%) *3 / 161*	0% (0 - 2.9%) *0 / 126*	0.12
Boopia bettongia	1.9% (0.7 - 5.3%) *3 / 161*	0% (0 - 2.9%) *0 / 126*	0.12
Flies:			
Lucilia sp. [4]	0% (0 - 2.3%) *0 / 161*	0.8% (0.2 - 4.3%) *1 / 126*	0.26
Calliphora sp. [4]	0% (0 - 2.3%) *0 / 161*	0.8% (0.2 - 4.3%) *1 / 126*	0.26

Parasite	Bushland % infected (95% CI) *No. infected / no. tested*	Urbanised environment % infected (95% CI) *No. infected / no. tested*	χ^2 test p-value[1]
Ticks:			
Family Ixodidae[5]	31.7% (25.0 - 39.2%) *51 / 161*	49.2% (40.6 - 57.8%) *62 / 126*	0.003
Ixodes spp.	10.6% (6.7 - 16.3%) *17 / 161*	37.3% (29.3 - 46.0%) *47 / 126*	< 0.001
Ixodes fecialis	7.5% (4.3 - 12.6%) *12 / 161*	36.5% (28.6 - 45.2%) *46 / 126*	< 0.001
Ixodes australiensis	7.5% (4.3 - 12.6%) *12 / 161*	6.3% (3.3 - 12.0%) *8 / 126*	0.72
Amblyomma spp.[4]	19.9% (14.5 - 26.7%) *32 / 161*	12.7% (8.0 - 19.7%) *16 / 126*	0.11
Haemaphysalis humerosa	5.6% (3.0 - 10.3%) *9 / 161*	0.8% (0.2 - 4.3%) *1 / 126*	0.028
Mites:			
Order Mesostigmata	61.5% (53.8 - 68.7%) *99 / 161*	56.3% (47.6 - 64.7%) *71 / 126*	0.38
Mesostigmatan morphotype 1[6]	59.6% (51.9 - 66.9%) *96 / 161*	56.3% (47.6 - 64.7%) *71 / 126*	0.58
Mesostigmatan morphotype 2[7]	17.4% (12.3 - 24.0%) *28 / 161*	4.0% (1.8 - 8.9%) *5 / 126*	< 0.001
Trombiculid mites (Suborder Prostigmata)	21.7% (16.1 - 28.7%) *35 / 161*	7.1% (3.8 - 13.0%) *9 / 126*	0.001

[1] χ^2 test comparing prevalences of infection in quenda trapped in urbanised environments compared to bushland

[2] Two fleas from two hosts were not able to be differentiated further morphologically, due to specimen damage

[3] Fifty *Pygiopsylla* spp. fleas from 22 hosts (host obtained from 20 sites) were differentiated to species level - all were *Pygiopsylla tunneyi*

[4] All specimens were larvae (flies) or larvae and nymphs (*Amblyomma* spp.)

[5] Ninety ticks from 25 hosts were not able to be differentiated further morphologically, due to specimen damage.

[6] Twenty five mites from 11 hosts (hosts obtained from ten sites) were differentiated to species level - 22 were *Haemolaelaps marsupialis*, two were *Gymnolaelaps annectans,* and one was *Mesolaelaps australiensis*.

[7] Twenty five mites from 10 hosts (hosts obtained from nine sites) were differentiated to species level - all were *Mesolaelaps antipodianus*

Table 7.5 Univariable logistic regression, investigating the association between the respective ectoparasite taxon and urbanisation in Perth quenda

Parasite taxon	Logistic regression type	Independent variable	Crude OR	95% CI	p-value
Pygiopsylla spp.	Generalised estimating equations	Bushland	1		
		Urbanised environment	2.32	0.92 – 5.90	0.075
Stephanocircus dasyuri	Generalised estimating equations	Bushland	1		
		Urbanised environment	1.31	0.28 – 6.21	0.74
Echidnophaga myrmecobii	Generalised estimating equations	Bushland	1		
		Urbanised environment	3.49	0.22 – 55.4	0.38
Echidnophaga gallinacea	Generalised estimating equations	Bushland	1		
		Urbanised environment	3.03	0.48 – 19.1	0.24
Ixodes fecialis	Generalised estimating equations	Bushland	1		
		Urbanised environment	5.10	1.39 – 18.7	0.014
Haemaphysalis humerosa	Generalised estimating equations	Bushland	1		
		Urbanised environment	0.16	0.01 – 2.65	0.20
Mesostigmatan morphotype 2	Generalised estimating equations	Bushland	1		
		Urbanised environment	0.35	0.076 – 1.62	0.18
Trombiculid mites (Suborder Prostigmata)	Generalised estimating equations	Bushland	1		
		Urbanised environment	0.34	0.060 – 1.90	0.22

Table 7.6 Multivariable logistic regression, investigating the association between the respective parasite taxon and urbanisation in Perth quenda

Age of the quenda, sex and season of trapping and sampling were considered as potential confounders

Parasite taxon	Logistic regression type	Independent variable	Adjusted OR	95% CI
Pygiopsylla spp.	Generalised estimating equations	Bushland	1	
		Urbanised environment	2.32[1]	0.92 – 5.90[1]
Ixodes fecialis	Generalised estimating equations	Bushland	1	
		Urbanised environment	6.34	1.58 – 25.5

[1] *Pygiopsylla* spp. - as per crude OR; no evidence of confounding by age, sex and / or season.
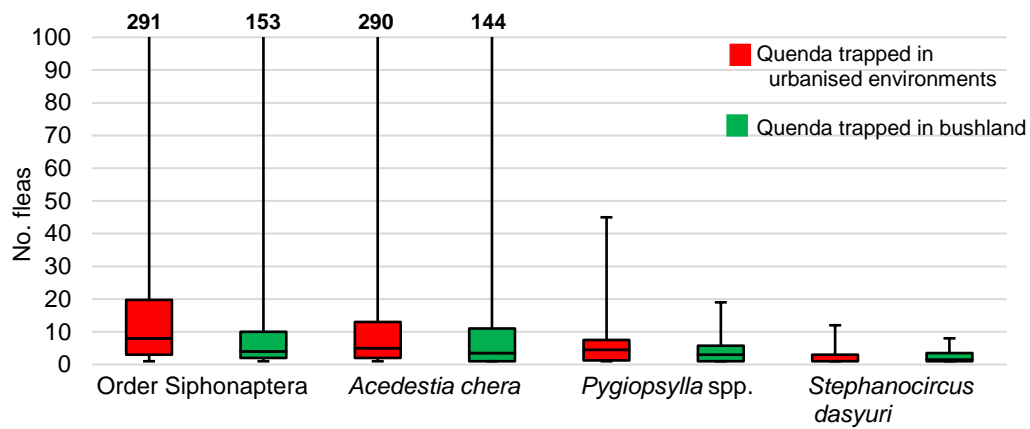
Figure 7.4 Intensity of flea infections in Perth quenda

Box and whisker plots presenting median, interquartile range and range of flea infection intensities. Upper whiskers of Order Siphonaptera and *Acedestia chera* intensities abbreviated with numbers, representing upper limit of respective intensities, to facilitate visualisation of all medians and interquartile ranges.
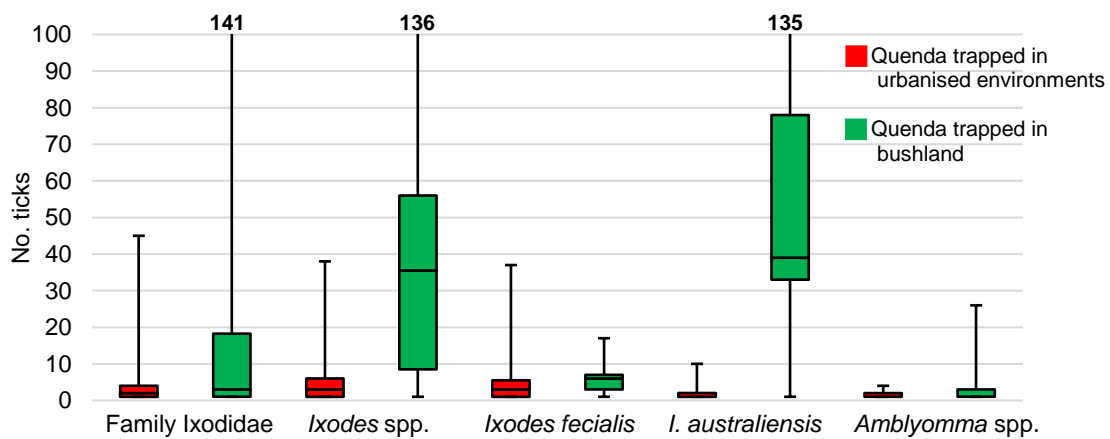
Figure 7.5 Intensity of tick infections in Perth quenda

Box and whisker plots presenting median, interquartile range and range of tick infection intensities. Upper whiskers of Family Ixodidae (bushland), *Ixodes* spp. (bushland) and *I. australiensis* (bushland) intensities abbreviated with numbers, representing upper limit of respective intensities, to facilitate visualisation of all medians and interquartile ranges.
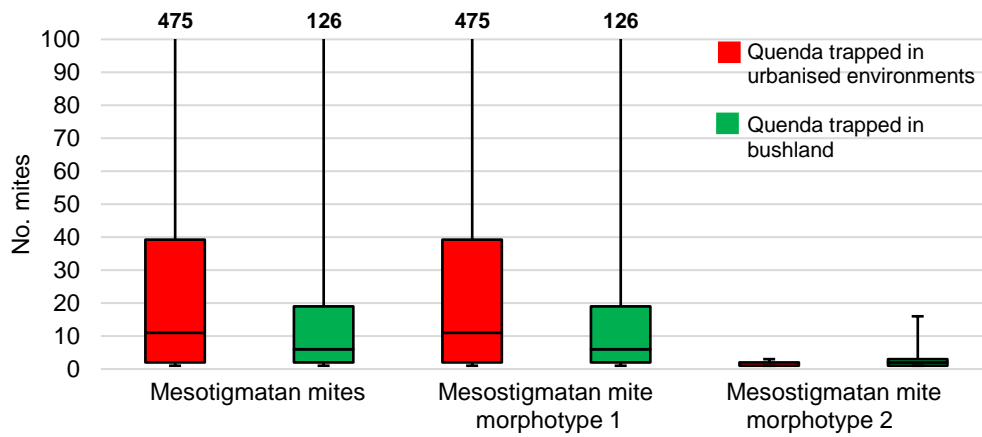
Figure 7.6 Intensity of mesostigmatan mite infections in Perth quenda

Box and whisker plots presenting median, interquartile range and range of mesostigmatan mite infection intensities. Upper whiskers of Mesostigmatan mites and Mesostigmatan mite morphotype 1 intensities abbreviated with numbers, representing upper limit of respective intensities, to facilitate visualisation of all medians and interquartile ranges.

Table 7.7 Intensities of ectoparasite infection in quenda trapped in urbanised environments and bushland in Perth

Parasite	Bushland median	Urbanised environment median	Wilcoxon rank sum test p-value
Fleas:			
Order Siphonaptera[1]	4	8	0.003
Acedestia chera	3.5	5	0.19
Pygiopsylla spp.[2]	3	4.5	0.015
Stephanocircus dasyuri	1.5	3	0.47
Ticks:			
Family Ixodidae[3]	3	2	0.077
Ixodes spp.	35.5	3	< 0.001
Ixodes fecialis	6	3	0.059
Ixodes australiensis	39	2	0.001
Amblyomma spp.[4]	1	1	0.47
Mesostigmatan mites:			
Order Mesostigmata	6	11	0.052
Mesostigmatan morphotype 1[5]	6	11	0.036
Mesostigmatan morphotype 2[6]	2	2	0.67

[1] Two fleas from 2 hosts were not able to be differentiated further morphologically, due to specimen damage

[2] Fifty *Pygiopsylla* spp. fleas from 22 hosts (host obtained from 21 sites) were differentiated to species level - all were *Pygiopsylla tunneyi*

[3] Ninety ticks from 25 hosts were not able to be differentiated further morphologically, due to specimen damage.

[4] All specimens were larvae or nymphs

[5] Twenty five mites from 11 hosts (hosts obtained from ten sites) were differentiated to species level - 22 were *Haemolaelaps marsupialis*, two were *Gymnolaelaps annectans,* and one was *Mesolaelaps australiensis.*

[6] Twenty five mites from 10 hosts (hosts obtained from nine sites) were differentiated to species level - all were *Mesolaelaps antipodianus*

Table 7.8 Univariable linear regression, investigating the association between the intensity of the respective ectoparasite taxon infection and urbanisation in Perth quenda

Parasite taxon	Linear regression type	Independent variable	Coefficient	95% CI	p-value
Pygiopsylla spp.[1]	Ordinary	Bushland	0		
		Urbanised environment	0.18	0.050 - 0.32	0.008
Ixodes fecialis	Generalised estimating equations	Bushland	0		
		Urbanised environment	-0.25	-0.53 - 0.025	0.075
Ixodes australiensis	Generalised estimating equations	Bushland	0		
		Urbanised environment	-1.22	-1.76 - -0.68	< 0.001
Mesostigmatan mite morphotype 1[2]	Ordinary	Bushland	0		
		Urbanised environment	0.24	0.023 - 0.43	0.029

[1] Adjusted R^2 = 0.042

[2] Adjusted R^2 = 0.023

Table 7.9 Multivariable linear regression, investigating the association between the intensity of infection with the respective parasite taxon and urbanisation in Perth quenda
Age of the quenda, sex and season of trapping and sampling were considered as potential confounders

Parasite taxon	Linear regression type	Independent variable	Adjusted coefficient	95% CI
Pygiopsylla spp.[1]	Ordinary	Bushland	0	
		Urbanised environment	0.23	0.088 - 0.36
Ixodes fecialis	Generalised estimating equations	Bushland	0	
		Urbanised environment	-0.21	-0.54 - 0.11
Ixodes australiensis	Generalised estimating equations	Bushland	0	
		Urbanised environment	-0.96	-1.57 - -0.35
Mesostigmatan mite morphotype 1[2]	Ordinary	Bushland	0	
		Urbanised environment	0.31	0.11 - 0.50

[1] Adjusted R^2 = 0.068

[2] Adjusted R^2 = 0.11

7.3.2   Quenda parasite infracommunity composition, in urbanised environments compared to bushland

All 284 quenda from which a faecal sample was obtained were infected with at least one gastrointestinal parasite taxa, and thus all were included in infracommunity composition analyses. Two hundred and twenty seven quenda (101 trapped in

urbanised environments and 126 trapped in bushland) were infected with at least one ectoparasite taxa, and thus were included in the ectoparasite infracommunity composition analyses.

There were differences in endoparasite and ectoparasite infracommunity composition in quenda in urbanised environments compared to bushland and across seasons, but not between sexes or maturity stages (Table 7.10). The parasite taxa making the most substantial contributions to these differences are listed in Tables 7.11 and 7.12.

Table 7.10 Analysis of similarities (ANOSIM) of the respective quenda parasite infracommunities, in urbanised environments compared to bushland, across seasons, between sexes and in subadult quenda compared to adults

Dependent variable	Independent variable	One-way ANOSIM		Two-way ANOSIM	
		Global R statistic	Significance level	Global R statistic	Significance level
Gastrointestinal parasite community	Urbanisation	0.084	0.001	0.10	0.001
	Season	0.083	0.001	0.12	0.001
	Sex	0.005	0.26	-	-
	Maturity	0.04	0.23	-	-
Ectoparasite community	Urbanisation	0.084	0.001	0.047	0.017
	Season	0.38	0.001	0.41	0.001
	Sex	0.016	0.76	-	-
	Maturity	0.042	0.25	-	-

Table 7.11 Parasite taxa contributing to the differences in composition of quenda gastrointestinal parasite infracommunities, between urbanised environments and bushland in Perth quenda

Contributing taxa	% Contribution
Linstowinema spp.	20.4%
Trichuris spp.	17.8%
Giardia spp.	15.7%
Labiobulura spp.	10.9%
Cryptosporidium spp.	9.04%
Strongyloids	8.07%
Potorolepis spp.	6.99%
Coccidia[1]	4.20%

[1] Family Eimeriidae

Table 7.12 Parasite taxa contributing to the differences in composition of quenda ectoparasite infracommunities, between urbanised environments and bushland in Perth

Contributing taxa	% contribution
Pygiopsylla spp.	15.8%
Acedestia chera	14.7%
Mesostigmatan mites morphotype 1	14.0%
Ixodes fecialis	12.4%
Amblyomma spp.	10.7%
Trombiculid mites	8.60%
Stephanocircus dasyuri	7.28%
Mesostigmatan mites morphotype 2	6.68%

7.3.3 Quenda morphometrics, in urbanised environments compared to bushland

Head length data were available from 112 / 117 (95.7%) adult females and 134 / 142 (94.4%) adult males. Average pes length was available from all sampled quenda. Scrotal width was available from 141 (99.3%) adult males. Net weight and thus also objective body condition were available from 141 (99.3%) adult males and 44 (37.6%) adult females (the missing adult female data was entirely due to the presence of pouch young). Subjective body condition was available from 258 / 259 (99.6%) adult quenda. Morphometric data are summarised in Figures 7.7, 7.8 and 7.9, and Table 7.13.
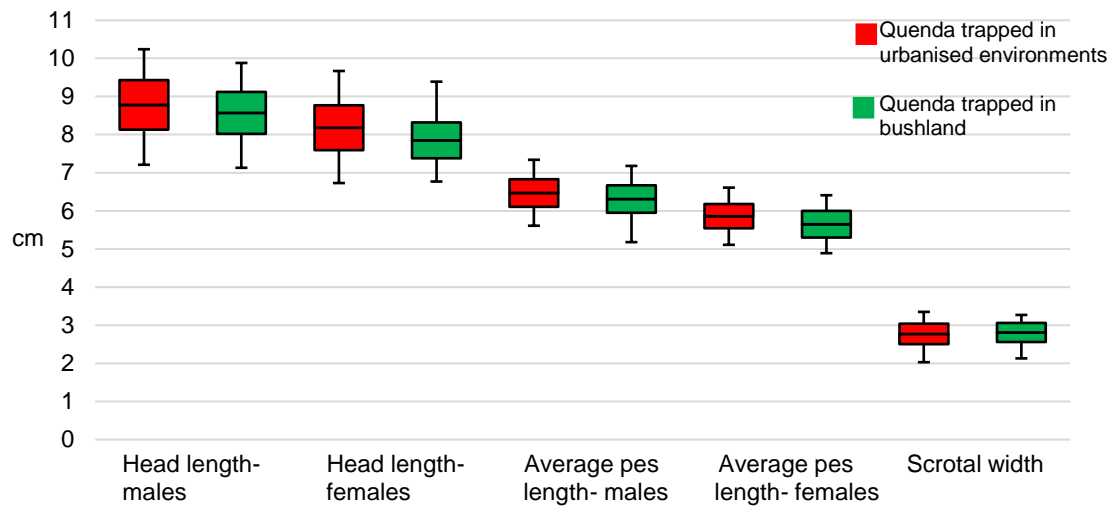
Figure 7.7 Morphometrics of adult quenda trapped in urbanised environments and bushland in Perth

Box and whisker plots presenting mean, upper and lower standard deviations, and range of the respective morphometrics.
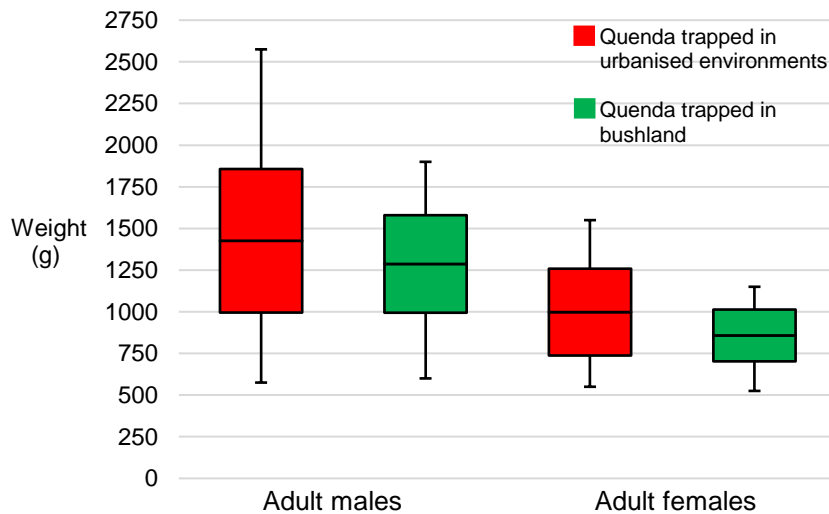
Figure 7.8 Net weight of adult quenda trapped in urbanised environments and bushland in Perth

Box and whisker plots presenting mean, upper and lower standard deviations, and range of net weights.
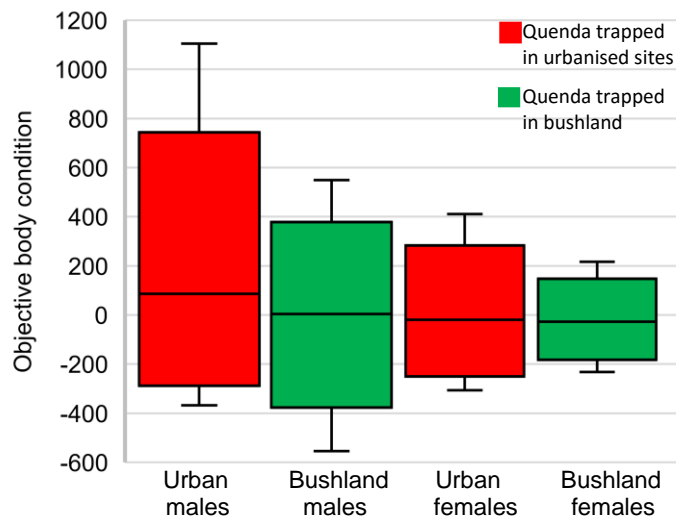
Figure 7.9 Objective body condition of adult quenda trapped in urbanised environments and bushland in Perth

Box and whisker plots presenting mean, upper and lower standard deviations, and range of objective body condition.

Table 7.13 Subjective body condition of adult quenda trapped in urbanised environments and bushland in Perth

| Trap site | Subjective body condition No. quenda (%) | | | | | |
	1 (emaciated)	2 (underweight)	3 (optimal)	4 (overweight)	5 (obese)	Total
Urbanised	0 (0%)	2 (1.9%)	47 (43.9%)	51 (47.7%)	7 (6.5%)	107 (100%)
Bushland	0 (0%)	13 (8.6%)	111 (73.5%)	27 (17.9%)	0 (0%)	151 (100%)

Urbanisation was associated with an increase in net weight of adult male quenda by 189.0 g (95% CI 68.56 – 309.47 g; p = 0.002; adjusted R^2 = 0.058) and an increase in net weight of adult females by 140.1 g (95% CI 3.91 – 276.3 g; p = 0.044; adjusted R^2 = 0.072). A skew towards higher body condition in urbanised areas was evident in both the objective and subjective body condition data (Figure 7.9 and Table 7.13). There was strong (p = 0.001) and very strong (p < 0.001) evidence of an association between urbanisation and subjective body condition in adult male and female quenda, respectively. There was also good evidence (p = 0.033) of an association between

urbanisation and objective body condition in adult male quenda (regression coefficient = 96.8, 95% CI 8.02 – 185.6; adjusted R^2 = 0.41). However, there was insufficient evidence (p = 0.86) of an association in adult females (regression coefficient = 7.86, 95% CI -83.4 – 99.1; adjusted R^2 = -0.023).

There was no evidence of an association between urbanisation and average pes length in adult males and females, or scrotal width in adult males (p > 0.10). However, there was weak evidence of an association between head length and urbanisation in both adult males (regression coefficient = 0.20, 95% CI -0.002 – 0.41; p = 0.052; adjusted R^2 = 0.021) and adult females (regression coefficient = 0.21, 95% CI -0.0035 – 0.42; p = 0.054; ICC = 0.26).

Where mixed effects linear regression models were used for the morphometrics analyses, the results were similar to the equivalent generalised estimating equations model (results not shown).

7.3.4 Reproductive status of adult female quenda, in urbanised environments compared to bushland

Of the 117 adult female quenda trapped in this study, 88 (75.2%) had an active pouch. Females with an active pouch were trapped in all months from March to November, inclusive. In autumn, the proportion of adult female quenda with an active pouch was higher in bushland than in urbanised environments (Table 7.14).

Table 7.14 The percentage of adult female quenda trapped with an active pouch, in urbanised environments and bushland in Perth, by season

	Bushland % active pouch (95% CI) *No. active pouch /* *total no. adult females*	Urbanised environments % active pouch (95% CI) *No. active pouch /* *total no. adult females*
Autumn	76.5% (52.4 - 90.3%) *13 / 17*	25.9% (13.2 - 44.9%) *7 / 27*
Spring	93.6% (82.8 - 97.7%) *44 / 47*	91% (72.0 - 97.2%) *20 / 22*
Winter	100% (47.8 - 99.5%) *4 / 4*	-

In the univariable analysis, there was good evidence of an inverse association between urbanisation and pouch activity (p = 0.009). Adult female quenda trapped in bushland had over eight and a half times the odds of having an active pouch than those trapped in urbanised environments (crude OR = 8.74; 95% CI 1.84 - 41.6). Season was retained in the multivariable model; consequently, the four adult female quenda trapped in winter were excluded from the multivariable model, as they were exclusively trapped in bushland. After controlling for season, adult female quenda trapped in bushland had just under five times the odds of having an active pouch, compared to quenda trapped in urbanised environments (adjusted OR = 4.89; 95% CI 1.65 - 14.46). There was no evidence of interaction between season and urbanisation (likelihood ratio test p = 0.12). The equivalent generalised estimating equations model provided very similar results to the mixed effects logistic regression model (results not shown).

7.4   Discussion

Urbanisation may affect disease ecology of wildlife, domestic animals and humans in a number of ways. Teasing apart these complex effects and establishing the impacts on both wildlife health and zoonotic risk requires more empirical data on host / parasite interactions in urban areas (Bradley and Altizer, 2007). In this study, we compared the parasite fauna, body condition and reproductive success of quenda, a common urban marsupial, in bushland and urbanised environments.

By comparing quenda trapped in bushland and urbanised environments within the same target population, potential bias in comparing quenda in urbanised environments to geographically remote bushland has been avoided. Such bias may occur through a raft of environmental variables that may influence parasite environmental persistence and baseline quenda population density, and the presence of sympatric native animal species that could influence the epidemiology of various parasites that can infect quenda, but have not been present in the greater Perth region for a long time, for reasons separate or additional to urbanisation (Kinnear et al., 2002). However, though it was evident that the quenda trapped in urbanised environments were exposed to urbanisation to a high degree, we cannot exclude the possibility that some quenda trapped in bushland also had home ranges that encompassed urbanised environments. Nevertheless, we believe that it would not have been typical of the quenda trapped in bushland to have had urbanised environments as a substantial proportion of their home range (Chapter 7.2), and we expect that any such misclassification would have biased our comparisons of quenda trapped in urbanised environments *vs.* bushland to the null. Therefore, identified differences between quenda trapped in urbanised environments *vs.* bushland remain valid, though possibly underestimated, but where no evidence against the null hypothesis was documented, Type II error attributable to such misclassification must be considered.

7.4.1 Parasites infecting free-ranging quenda in Perth, and comparisons of parasite prevalences and intensities of infection between quenda trapped in urbanised environments and bushland

This study documents a wide range of endo- and ecto- parasite taxa infecting quenda in Perth, and provides the first documentation of the fleas *Echidnophaga gallinacea* and *E. myrmecobii*, the flies *Lucilia* sp. and *Calliphora* sp., and the ticks *Ixodes australiensis*, *Amblyomma* spp. and *Haemaphysalis humerosa*, parasitising quenda.

Most of the gastrointestinal parasite taxa and ectoparasite taxa identified were found in quenda in both urbanised environments and bushland, and though there was preliminary evidence of differing prevalences between these environments, the correlated nature of the data is likely to have increased the risk of Type I error in these findings, as the differing prevalences were often not supported by evidence of differing odds of infection between the sites on logistic regression. However, there was evidence of differing odds of infection or intensity of infection in some parasite taxa between quenda trapped in urbanised environments and bushland. There were also differences in composition of parasite infracommunities between quenda trapped in urbanised environments and bushland. These differences may reflect different opportunities for transmission of particular parasites in different localities, which may relate to differences in the presence of alternative host species or, in some cases, intermediate host species, in urbanised environments compared to bushland, or different quenda population characteristics in urbanised environments, such as differing population densities.

7.4.1.1 Gastrointestinal protozoans and helminths

The protozoan genus *Giardia* spp. was more prevalent in urbanised environments than bushland, and this was supported by increased odds of infection in quenda trapped in urbanised environments compared to bushland after controlling for age, sex and season. This could be because of anthropogenic sources of infection or other factors such as quenda population density, which our subjective observations during trapping suggested were consistently high to very high in urbanised trapping sites, whilst varying widely in the bushland trapping sites.

Both *Giardia* spp. and *Cryptosporidium* spp. require molecular characterisation to differentiate the host-adapted species from anthropozoonotic species, to ascertain whether quenda are likely to be acquiring these infections from humans and domestic animals, and whether they are acting as a reservoir of infection of public health

significance. The findings of Teichroeb et al. (2009) and Johnston et al. (2010) suggest that *Giardia* spp. transmission can occur between populations of humans, livestock and non-human primates in Ghana and Uganda, where habitat usage overlaps. In contrast, Hill et al. (2008) found brushtail possums in urbanised environments in Sydney, Australia, were only infected with genetically novel *Cryptosporidium* spp. infections that have not been described infecting humans or domestic animals, suggesting that transmission of *Cryptosporidium* spp. between humans, domestic animals and brushtail possums is not common in that location. The zoonotic potential of *Cryptosporidium* spp. isolated from bandicoots (Family Peramelidae) in Sydney remains unresolved (Dowle et al., 2013). Anecdotally, the unusual morphology of the *Cryptosporidium* spp. on immunofluorescence microscopy noted in many samples of the present study corresponds with previous observations by others of novel Australian marsupial *Cryptosporidium* spp. morphology by light microscopy (Hill et al., 2008; A. Elliot, Murdoch University - pers. comm.). The *Entamoeba* spp. and coccidian oocysts observed in this study are not considered of public health significance, given the morphological findings and sporulation study findings (Chapter 10), respectively.

Of the nematode and cestode taxa found in this study, *Linstowinema* spp. and *Labiobulura* spp. are nematode genera of native marsupials and are not known to infect humans or pets. The strongyles, strongyloids and *Trichuris* spp. are considered likely to be native helminth species, given the genera of strongyles and strongyloids, and the species of *Trichuris*, identified in helminth screens of 49 quenda sampled post mortem from the same geographical area (A. Hillman, unpublished data). It is also considered likely that the *Physaloptera* spp. eggs identified are of a native species (e.g. *Physaloptera peramelis*), but this cannot be determined based on faecal egg morphology alone. *Potorolepis* spp. is a cestode genus of native marsupials, and has not been shown to parasitise domestic animal hosts.

Linstowinema spp. and *Physaloptera* spp. were more prevalent in urbanised environments, and this was supported by increased odds of infection in quenda trapped in urbanised environments compared to bushland. Population density, or in the case of *Physaloptera* spp. varying distribution of the (currently unknown) intermediate host species, may explain these discrepancies.

The unidentified oxyurids are likely to be artefactual findings from quenda prey items or incidentally ingested whilst foraging, although the possibility that they represent an as-yet undescribed oxyurid parasite of quenda cannot be excluded.

The presence of *Ascaridia* spp*.* eggs in the faeces of five quenda trapped in urbanised environments are also considered an artefactual findings regarding quenda parasitism. However, it raises the possibility that quenda in urbanised environments could act as vectors of this genus of parasites, although we were not able to assess the viability of the eggs due to preservation of faecal samples in formalin prior to analysis. If so, this is of relevance to health management of strategies of domestic poultry sharing their environment with quenda.

Misclassification bias is unlikely to have substantially impacted the *Giardia* spp. prevalence estimate obtained by immunofluorescence microscopy, but the faecal flotation protocol may have underestimated the prevalences of gastrointestinal helminths, particularly *Potorolepis* spp. (Chapter 4), and may not have been capable of detecting helminths with relatively heavy eggs. The accuracy of the respective tests used in the detection of *Cryptosporidium* spp., *Entamoeba* spp. and coccidian oocysts in quenda has not been ascertained, so likely impacts of misclassification are unclear.

7.4.1.2   Ectoparasites

The overwhelmingly predominant flea taxa identified in this survey, in terms of prevalence and intensity of infection, were the native fleas *Acedestia chera*,

Pygiopsylla spp. and *Stephanocircus dasyuri*. These taxa have never or rarely been

identified infecting domestic animal hosts. The tendency to increased intensity of

Pygiopsylla spp. infections in quenda trapped in urbanised environments may be a

result of relatively increased population density - associations have been demonstrated

between intensity of flea infection and host population density in other host species

(e.g. Krasnov et al., 2002; Kaal et al., 2006). *Pygiopsylla* spp. are presumptive fur fleas,

so transmission frequency would be expected to be positively related to host contact

rates. By contrast, it is suspected that *Acedestia chera* is a nest flea (Dunnet and

Mardon, 1974), and this may explain why it did not show a difference in intensity of

infection between urbanised and bushland environments. *Stephanocircus dasyuri* was

relatively rare, and a lack of power may explain why there was no evidence of

increased intensity of infection in urbanised environments, as per *Pygiopsylla* spp.

Echidnophaga gallinacea is a domestic poultry parasite that is capable of infecting a

wide range of mammals and birds, including other Australian marsupial species and

domestic animals (Dunnet and Mardon, 1974). Though this study did not find sufficient

statistical evidence of an association between urbanisation and the odds of infection

with *E. gallinacea*, most infected quenda were trapped in urbanised environments and

this lack of statistical support probably reflects a lack of power. Exposure to domestic

animal sources of infection in urbanised environments is a likely important source of

infection. Quenda in Perth are known to enter poultry enclosures and scavenge poultry

food, and are present on private properties with domestic animals (Chapter 3; Howard

et al., 2014). As for the *Ascaridia* spp. egg findings, these findings indicate the need for

private property owners to be mindful of management strategies to control parasite

burdens in their poultry and pets where quenda are known or suspected to occur on

their property. This has implications for the health of their poultry and pets, of quenda,

and of other neighbourhood poultry and pets, given the possibility that quenda could

act as vectors of infection between properties. *Echidnophaga myrmecobii* has been

documented on a variety of Australian marsupial and domestic animal hosts (Dunnet and Mardon, 1974; Thomasz, 2014; Webster et al., 2014).

Boopia bettongia, the only louse taxon identified, parasitises various Australian marsupial species (von Kéler, 1971). It has not been documented infecting domestic animal hosts. The fly genera *Lucilia* sp. and *Calliphora* sp. can infest domestic pets, but quenda do not represent significant reservoirs to humans and domestic pets given the lifecycle and nature of infection with these genera.

Ixodes fecialis and I. australiensis are native species of ticks, and have been recorded infecting domestic animal hosts in a few cases (Roberts, 1970) but are not considered important species of ticks regarding potential domestic animal and human infestation (Barker and Walker, 2014). Quenda in urbanised environments had increased odds of infection with *Ixodes fecialis*, whilst quenda trapped in bushland had a tendency towards increased intensity of infection with *Ixodes australiensis*. Differences in the availability of other host species involved in the tick lifecycle (e.g. kangaroos), and quenda population density, may be contributing factors to these findings. A lack of power may explain why there was no evidence of differing *I. fecialis* intensity of infection in quenda trapped in urbanised environments *vs.* bushland. However, given the limited amount of information available regarding the biology of native Australian tick species that do not commonly infect humans or domestic animals, it is difficult to speculate why urbanisation was associated with *I. fecialis* prevalence, supported by differing odds of infection on logistic regression, whilst it was not associated with *I. australiensis* prevalence.

Haemaphysalis humerosa is a native species of tick, and its exceptionally restricted range was an unusual finding. The bushland reserve in which *H. humerosa* was prevalent is known to have a number of sympatric native species populations that are not common in other bushland areas within the greater Perth region. However,

previous research suggests that bandicoots (Family Peramelidae, the taxonomic family of quenda) are primary host species for this tick (Roberts, 1963), and therefore the presence of other (non-peramelid) host species would not seem to be a plausibly important influence on *H. humerosa* distribution.

It is not possible to determine the anthropozoonotic significance of the *Amblyomma* spp. ticks, without knowing the particular species infecting quenda.

The species of mesostigmatan mites identified in this study have occasionally been identified on rabbits, hares and variety of native animals (Domrow, 1987), but are considered unlikely to infest other domestic animals or humans. Quenda in urbanised environments may have had a tendency to high intensity of infection with mesostigmatan mite morphotype 1 as a result of population density. Mesostigmatan mite morphotype 2 was much less prevalent, and therefore the analyses would have been comparatively low in power, which may explain the discordant findings between the two morphotypes. Trombiculid mites would require further taxonomic differentiation to clarify their host range.

It is possible that the ectoparasites identified on quenda are capable of acting as vectors of other infections of relevance to veterinary public health. However, this is an area in need of further investigation, as current knowledge of infections in quenda ectoparasites, and their ability to infect domestic animals and humans, is very limited.

Misclassification and measurement error (respectively) may have influenced ectoparasite prevalence and intensity estimates, but such bias is expected to be non-differential between urbanised environments and bushland. However, though some fleas and mesostigmatan mites and are associated with animal nests, and therefore screening an animal does not account for nest ectoparasites, Krasnov et al. (2004)

demonstrated that sampling both 'fur' fleas and 'nest' fleas of various species on various hosts correlated well with the nest burdens of the respective species.

In comparison to our findings, long-nosed bandicoots trapped in backyards and in one bushland site in the greater Sydney region, Australia, were found to have higher tick (Order Ixodida) prevalences that those trapped in a second bushland site, whilst all other ectoparasite prevalences were similar across the sites (Dowle, 2012). These findings were not further investigated with analyses that considered the effect of data correlation or confounding.

7.4.1.3 *Toxoplasma gondii* seroprevalence

The prevalence of *Toxoplasma gondii* antibodies was low in both bushland and urban quenda (Table 7.1). Although research suggests that this test is highly specific as an indicator of *T. gondii* infection in quenda (Chapter 4), the sensitivity has not been ascertained so this estimation needs to be interpreted with caution regarding inferring infection prevalence. While a large number of animals were sampled from both bushland and urbanised sites (n = 104 and 54, respectively) sampling was incomplete as jugular venesection proved difficult in quenda, particularly in urban areas where quenda were more obese. The jugular groove is not discernible, the narrow gauge needles used were prone to blockage, and sampling attempts are limited by ethical considerations. Ultrasound guidance was not used, but could improve such sampling success in future research.

In contrast to our findings, Lehrer et al. (2010) found *T. gondii* seroprevalence relatively common in woodchucks (*Marmota monax*) in Illinois, USA, and findings suggested that woodchucks inhabiting more urbanised environments had a greater likelihood of seropositivity.

7.4.2 Quenda parasite infracommunity composition, in urbanised environments compared to bushland

As expected from the comparison of odds of infection in individual parasite taxa, *Linstowinema* spp., *Trichuris* spp. and *Giardia* spp. all made important contributions to the differences in endoparasite community composition between quenda trapped in urbanised environments and bushland, while *Ixodes fecialis* was an important contributor to the differences in ectoparasite community composition between quenda trapped in urbanised environments and bushland. Unexpectedly, *Labiobulura* spp., which was highly prevalent in quenda trapped in both urbanised environments and bushland, was also an important contributor to differences in endoparasite community composition, while *Acedestia chera*, mesostigmatan mites morphotype 1 and *Amblyomma* spp., also highly prevalent in all sites, were important contributors to differences in endoparasite community composition. This reflects the importance of highly prevalent taxa in the analysis of community composition; even small differences in prevalence may contribute substantially to differences in composition if these taxa dominate the community structure.

7.4.3 Quenda morphometrics, in urbanised environments compared to bushland

Adult quenda trapped in urbanised environments tended to be heavier than those in trapped in bushland in Perth. The relatively increased weight of quenda in urbanised environments is consistent with the findings of Harrison (1997) regarding grey foxes (*Urocyon cinereoargenteus*) in New Mexico, USA; Cypher and Frost (1999) regarding San Joaquin kit foxes in California, USA; Harveson et al. (2007) regarding Key deer (*Odocoileus virginianus clavium*) in Florida Keys, USA; and Cahill et al. (2012) regarding wild boar in Barcelona, Spain. Our finding is likely due to a tendency towards obesity in adult quenda in urbanised environments. The overall picture of a relative increase in weight, in the absence of convincing evidence for an increase in skeletal morphometrics, in quenda trapped in urbanised environments compared to bushland,

with both the subjective and objective body condition data indicating a skew towards higher condition in quenda trapped in urbanised environments, supports this conclusion. Though both the subjective body condition and objective body condition measurements are prone to various biases as measures of body condition and lack validation in quenda, and the objective body condition is further limited by a lack of power, the overall combination of findings remain convincing. Similar to our findings, long-nosed bandicoots trapped in suburban backyards in Sydney, Australia, were found to have higher body condition than those trapped in bushland within the greater Sydney region (Dowle, 2012).

If obesity is the cause of the weight difference between bushland and urbanised environments, this may be attributable to access to anthropogenic food sources in urbanised environments. Quenda are opportunistic feeders (Broughton and Dickman, 1991), and in Perth are known to have access to pet food and compost heaps, and are sometimes offered food by members of the public (Chapter 3; Howard et al., 2014). Alternatively, relatively abundant resources in urbanised environments may be allowing quenda to grow larger in accordance with inherent genetic potential. Previous research supports such "phenotypic plasticity" in quenda (Hale, 2000) and in *Isoodon auratus* (Dunlop, 2015), a species which is genetically so closely related to quenda that conspecific status is justified (Pope et al., 2001; Zenger et al., 2005; Cooper et al., 2015). Other research has identified anthropogenic food sources as important resources to various urban dwelling / urban utilising mammals, such as gray foxes (*Urocyon cinereoargenteus*) in New Mexico, USA (Harrison, 1997), raccoons (*Procyon lotor*) in Chicago, USA (Bozek et al., 2007) and wild boar in Barcelona, Spain (Cahill et al., 2012).

7.4.4 Reproductive status of adult female quenda, in urbanised environments compared to bushland

This study suggests that female quenda in bushland may have increased fecundity, compared to those in urbanised environments, as represented by their odds of having an active pouch. Results by season suggest that the discrepancies in the odds of pouch activity between these groups is likely due to the relatively increased capacity of quenda in bushland sites to breed in autumn. The lack of statistical evidence of interaction between season and the association between urbanisation and pouch activity may have been attributable to a lack of power, considering the combination of a relatively small sample size and the inherent lack of power in statistical tests for interaction.

Quenda / southern brown bandicoots have an oestrous in late lactation, and therefore if optimally fertile are capable of having an active pouch continuously, or continuously throughout breeding season in populations where breeding occurs seasonally (Heinsohn, 1966; Stoddart and Braithwaite, 1979). Our results indicate that quenda in the greater Perth region are capable of breeding from at least March to November, inclusive (we did not undertake trapping in December, January or February). The ability of Perth quenda to breed in autumn corresponds to findings of Copley et al. (1990) and Sanderson and Kraehenbuehl (2006) of southern brown bandicoots in South Australia, which form an *I. obesulus* phylogenetic group with Western Australian quenda (Cooper et al., 2015). Research suggests that southern brown bandicoot populations from other eastern Australian locations, which form a separate phylogenetic group of *I. obesulus* (Cooper et al., 2015), breed seasonally with autumn not part of the breeding season (Heinsohn, 1966, Stoddart and Braithwaite, 1979; Lobert and Lee, 1990; Dowle, 2012).

Adult female quenda trapped in urbanised environments in autumn in our study, however, often did not have an active pouch. These findings raise the possibility that reproductive capacity of adult female quenda is adversely affected by urbanisation,

with the effect possibly modified by season. This may be associated with our subjective observation of consistently high to very high quenda population densities in trapping urbanised sites. Given that quenda have a high reproductive potential, it has been speculated that they may have an intrinsic population regulation mechanism induced by stress associated with high population density (Thomas, 1990). Harveson et al. (2007) similarly found evidence of relatively decreased reproduction in Key deer (*Odocoileus virginianus clavium*) associated with increased urbanisation, in only two seasons of the year (with comparable reproduction in the other two seasons), and also believed it to be a density-dependent response to the population reaching carrying capacity. However, these findings contrast to that of Statham and Statham (1997), who found brushtail possums in an urbanised environment in Tasmania, Australia, had births throughout the year, in contrast to the seasonal breeding of non urbanised Tasmanian brushtail possum populations. This was attributed to abundant nutritional resources available in urban areas. Similarly, Gould and Andelt (2011) found female San Clemente Island foxes (*Urocyon littoralis clementae*) that spent greater proportions of time within urban areas had a greater probability of reproducing.

Other possible factors that could negatively impact fertility of adult female quenda in urbanised environments in Perth include: 1) a tendency towards obesity in quenda in urbanised environments impacting fertility; 2) higher prevalence of infections that impact fertility in urbanised environments; or 3) a longer lifespan of quenda in urbanised environments resulting in relatively decreased population fertility (an association between urbanisation and survivability has been documented in white-tailed deer (*Odocoileus virginianus*) in Chicago, USA (Etter et al., 2002); raccoons in Illinois, USA (Prange et al., 2003); Key deer in Florida Keys, USA (Harveson et al., 2007); and woodchucks (*Marmota monax*) in Illinois, USA (Watson, 2009). However, it does not seem plausible that these factors would have effects modified by season. Studies involving temporal sequence are required to clarify the significance of these findings.

7.5 Conclusions

Urbanisation is increasing rapidly on a global scale, but the effects of this increase on wildlife health are poorly studied. We found that urbanised environments were associated with increased body mass in quenda, which may be attributable to higher levels of obesity in urbanised environments compared to bushland. Adult female quenda in urbanised environments appear to have reduced fecundity, with the effect possibly modified by season. Perth quenda are commonly parasitised by a variety of endo- and ecto- parasites, but these are overwhelmingly of native parasite taxa not known to infect humans or domestic pets. However, the identified *Giardia* spp. and *Cryptosporidium* spp. infections are of potential anthropozoonotic significance and this requires further clarification (Chapter 8). There are variations in the odds of infection or intensity of infection with certain parasites, and in the composition of parasite communities, between quenda trapped in urbanised environments compared to bushland, which are likely explained by differences in transmission potential driven by quenda population density and the existence or otherwise of alternative host species. This study provides the first documentation of several ectoparasite taxa (*Echidnophaga gallinacea and E. myrmecobii, Lucilia* sp., *Calliphora* sp., *Ixodes australiensis, Haemaphysalis humerosa* and *Amblyomma* spp.) parasitising quenda. Of these, *Echidnophaga gallinacea* is of particular interest because it is a domestic poultry parasite that is capable of infecting a wide range of birds and mammals, with potential adverse consequences including infection transmission. It provides a good example of the potential threats that urbanisation poses for the health of wildlife and domestic species when caused to cohabitate.

7.6 Acknowledgements

7.7 References

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Chapter 8

Anthropozoonotic significance, risk factors and spatial distribution of *Giardia* spp. infections in quenda (*Isoodon obesulus*) in the greater Perth region, Western Australia

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8. Anthropozoonotic significance, risk factors and spatial distribution of *Giardia* spp. infections in quenda (*Isoodon obesulus*) in the greater Perth region, Western Australia

8.1 Introduction

The epidemiology of *Giardia* spp. infection in wildlife, particularly in urban and peri-urban areas, is of interest for two reasons. Firstly, wildlife species have been shown to be susceptible to infection with *Giardia* spp. from humans and domestic pets, and thus may act as a reservoir of infection of public health significance. Secondly, the impacts of human and domestic animal-based infections (including *Giardia* spp.) on wildlife population health are of concern, not only for urban dwelling wildlife populations (Kutz et al., 2009; Thompson, 2013), but also regarding urban to non-urban translocation of wildlife, because translocated animals have the potential to act as vectors to spread human and domestic animal-based infections to wildlife populations which may not otherwise be exposed to such pathogens (Cunningham, 1996).

In Perth, Western Australia, quenda (syn. southern brown bandicoots, *Isoodon obesulus*) exist in many urbanised areas. They are small, terrestrial marsupials, and turn over up to four tonnes of soil per year each in foraging for food (Valentine et al., 2013). Thus, they are considered to be at particular risk of acquiring soil-transmitted parasites such as *Giardia* spp., and in urbanised environments are also at risk of exposure to pathogens originating from domestic pet faeces, and human faeces via septic tanks. Quenda are known to be (illegally) translocated from private properties in the greater Perth region (Howard et al., 2014), and so may be both at risk of adverse impacts of anthropozoonotic infections and potentially acting as a vector of such infections into non-urban populations.

We previously identified a high prevalence of *Giardia* spp. infection in quenda in the greater Perth region, Western Australia (Chapter 7). We aimed to expand on this finding, by:

1) using PCR and sequencing to identify and measure the prevalence of the species of *Giardia* infecting Perth quenda;

2) identifying risk factors for *Giardia* spp. infection in Perth quenda, with a focus on environmental variables, demographic variables and putative influences of concurrent parasitic infections;

3) investigating geographical clustering of *Giardia* spp. infection risk in Perth quenda, particularly associated with a gradient of urbanisation; and

4) identifying associations between *Giardia* spp. infection and indicators of ill health in Perth quenda.

8.2 Methods

8.2.1 Trapping and sampling quenda

A cross sectional study of free-ranging quenda in urbanised environments and bushland in the Statistical Division of Perth was undertaken across March 2013 to July 2015. Twenty-nine bushland sites and 35 urbanised sites were trapped: site selection, and trapping and sampling of quenda, were undertaken using methods described in Chapter 7.

On collection from the trap, faecal samples from quenda were classified as 'formed' if at least one discrete faecal pellet was present, or 'poorly formed' if no discrete faecal pellets were present. Fur condition of sampled quenda was graded either as 'full coat', if the animal had complete fur cover, or 'incomplete coat' if there were areas of shearing, regrowth or alopecia present. Skin condition of the quenda was graded as 'normal' if there was no evidence of gross pathology, 'scale' if scale was present, or 'exudative' if there were exudative lesions present. Body condition was assessed via

two methods: 'subjective body condition' and 'objective body condition'. For the 'subjective body condition', fat and muscle mass were qualitatively palpated over the temporal fossa, scapula spine and spine: animals were then graded as: low condition (emaciated or underweight), optimal condition, or high condition (overweight or obese). The 'objective body condition' was calculated separately for adult male and adult female quenda, as the residual of observed to expected weight. Expected weight was calculated by linear regression of average pes length to net weight, using Stata 14 software (StataCorp, 2015), as described in Chapter 7.

Immunofluorescence microscopy of faeces was used to identify quenda infected with *Giardia* spp., using methods described in Chapter 7. In faecal samples positive for *Giardia* spp. on immunofluorescence microscopy, species differentiation was attempted using PCR and sequencing at the 18S rRNA, ITS1-5.8s-ITS2 and *gdh* loci, using methods described in Chapter 9.2. Quenda were considered positive for any species of *Giardia* if positive on immunofluorescence microscopy; quenda were considered positive for a particular species of *Giardia* if positive on immunofluorescence microscopy, with the species identified on PCR and sequencing at one or more loci.

Concurrent *Cryptosporidium* spp. infections were identified using immunofluorescence microscopy, using a commercial kit (Merifluor Cryptosporidium / Giardia, Meridian Bioscience, Inc. USA), according to manufacturer's directions for unconcentrated faecal samples. Samples were considered positive if at least one cyst of appropriate *Cryptosporidium* spp. morphology and fluorescence was identified.

Concurrent gastrointestinal protozoan and helminth parasites were tested for using a faecal flotation protocol (as described and validated in Chapter 4). Quenda were considered infected if at least one protozoan oocyst or helminth egg of the respective taxa was identified using this protocol.

All macroscopic ectoparasites on sampled quenda were collected and differentiated, as described in Chapter 7. Quenda were considered infected with an ectoparasite taxon if at least one specimen was obtained from the quenda, and differentiated as such microscopically.

8.2.2 *Giardia* spp. prevalence

Prevalences were calculated with Jeffrey's 95% confidence interval. *Giardia* spp. prevalence was mapped using ArcGIS 10.3.1 (Esri, 2015), using the GPS point of the trap in which the quenda was caught. On mapping, GPS points were jittered by 0.00001 decimal degrees, to allow better visualisation of the relative distribution of *Giardia* spp. infection in the sampled quenda.

8.2.3 *Giardia* spp. in quenda - risk factors for infection

Two environmental variables were assessed for association with *Giardia* spp. infection: 1) the season in which the quenda was trapped and sampled; and 2) whether the quenda was trapped in bushland or an urbanised environment. Bushland was defined as such if included in the Western Australian Planning Commission's Bush Forever policy (Department of Planning, 2000), however quenda trapped within a 200 m radius of an urbanised centre of bushland were defaulted to urbanised. Urbanised environment was defined as a private residential or non-residential property, or within 200 m of an urbanised centre of bushland (offices, with food outlets and barbecue areas). As per Chapter 7.2 and 7.4, trapping was undertaken with the aim of minimising the likelihood that quenda trapped on bushland sites also had urbanised environments as part of their home range.

Demographic variables assessed for association with *Giardia* spp. infection included: the sex of the animal; whether the animal was an adult (females - had a parous pouch; males - scrotal width ≥ 2cm) or subadult (females - had a non-parous pouch; males -

scrotal width < 2cm); and in adult females, whether the quenda had an active pouch (lactating) or an inactive pouch (not lactating).

Concurrent parasitic infections assessed for association with *Giardia* spp. infection included the range of gastrointestinal protozoan and helminth taxa, and the range of ectoparasite taxa, identified infecting the quenda. All taxa were assessed as present / absent, except fleas (Order Siphonaptera), ticks (Family Ixodidae) and mesostigmatan mites (Order Mesostigmata), where burden was assessed on an ordinal scale (nil = no specimens obtained; low = 1 to 9 specimens obtained; high ≥ 10 specimens obtained).

Logistic regression was undertaken using Stata 14 software (StataCorp, 2015) to assess for association between *Giardia* spp. infection and the independent variables. Prior to univariable analyses, each independent variable was tested for spatial autocorrelation, using trap site as a random effect. Where the likelihood ratio test p > 0.05, data were considered independent and analysed using standard logistic regression; where likelihood ratio test p ≤ 0.05, mixed effects logistic regression was undertaken. The only evidence of clustering by trap site occurred with *Entamoeba* spp. - this association was tested using mixed effects logistic regression, including trap site as a random effect. The validity of the mixed effect logistic regression model was checked using the quadrature check, and by comparing the model output to that of the equivalent generalised estimating equations model (utilising an exchangeable correlation matrix and robust standard errors), as described in Chapter 7.2.4. Results were reported with Wald p-values.

Multivariable models were built, considering all independent variables where on univariable analyses $p \leq 0.20$. Independent variables were sequentially added to the model - they were retained if they improved model fit (likelihood ratio test $p \leq 0.20$), and omitted from the multivariable model if they did not (likelihood ratio test $p > 0.20$). Testing for interaction between the independent variables was omitted from the multivariable model, due to the large number of independent variables constituting the final model. Independent variables were considered associated with *Giardia* spp. infection where the adjusted odds ratio 95% confidence interval did not cross the null value (adjusted OR = 1.00).

8.2.4 Geographical clustering of *Giardia* spp. infection risk

Geographical clustering of *Giardia* spp. infection in quenda in urbanised environments was initially investigated using logistic regression, as described in section 8.2.3. Kulldorff's spatial scan statistic (SaTScan version 9.4, www.satscan.org) was used to further investigate geographical clustering. Data were run as an unfocused Bernoulli model, scanning for clusters of both increased and decreased risk of *Giardia* spp. infection, utilising Gumbel approximation in the significance testing. The two most likely clusters, as determined by the p-value, were reported in the results and mapped using ArcGIS 10.3.1 (Esri, 2015).

8.2.5 Associations between *Giardia* spp. infection and indicators of ill health

Association between *Giardia* spp. infection and faecal condition, fur condition, skin condition and subjective body condition were tested by logistic regression, and association between *Giardia* spp. infection and objective body condition by linear regression, using Stata 14 software (StataCorp, 2015). On univariable analyses, each independent variable was tested for spatial autocorrelation, using trap site as a random effect. If likelihood ratio test $p > 0.05$, data were considered independent and standard

logistic regression was undertaken. For the linear regression, normal distribution of the model residuals was confirmed to ensure the models were valid.

8.3 Results

Two hundred and eighty seven quenda were trapped and sampled across 29 urbanised sites and 22 bushland sites (Figure 7.2). Three quenda (1 adult female and 2 adult males) were excluded from this study, as faeces were not recoverable from their traps. The 284 quenda included in this study comprised 11 subadult females, 17 subadult males, 116 adult females and 140 adult males. Of the adult females, 28 had an inactive pouch, and 88 had an active pouch.

8.3.1 *Giardia* spp. prevalence

On immunofluorescence microscopy, 99 / 284 of quenda were infected with *Giardia* spp. (34.9%; 95% CI 29.6 – 40.6%). Sixty-three of the *Giardia* spp. infections were successfully differentiated to species level by PCR and sequencing. All 63 of these quenda were confirmed to be infected with *G. peramelis* (22.2%; 95% CI 17.7 – 27.4%). One quenda was also infected with *G. duodenalis* (0.4%; 0.1 – 1.9%), and another was also infected with *G. canis* genotype D (0.4%; 95% CI 0.1 – 1.9).

8.3.2 *Giardia* spp. in quenda - risk factors for infection

On univariable analyses, both urbanisation and the season in which the quenda were sampled were associated with the odds of *Giardia* spp. infection ($p < 0.10$) (Table 8.1). No demographic variables were associated ($p > 0.10$) (Table 8.1). Regarding putative influences of concurrent parasitic infections, several taxonomic groupings of parasites were at least weakly associated ($p \leq 0.10$) with increased odds of *Giardia* spp. infection (Tables 8.2 and 8.3). This included the endoparasites *Cryptosporidium* spp., *Labiobulura* spp., *Linstowinema* spp. and *Trichuris* spp., and the ectoparasites fleas and mesostigmatan mites.

The *Entamoeba* spp. mixed effect logistic regression model output closely matched the equivalent generalised estimating equations model (results not shown), and all relative differences in the quadrature check were < 0.01.

Table 8.1 Univariable risk factors for *Giardia* spp. infection in Perth quenda - environmental and demographic variables

Variables with an association considered strong enough to be included in multivariable analysis are shown in bold.

		Crude OR[1]	95% CI	p-value
Trapping site	**Bushland**	**1**		
	Urbanised	**1.78**	**1.08 - 2.91**	**0.022**
Season	**Autumn**	**1**		
	Spring	**2.30**	**1.35 - 3.91**	**0.002**
	Winter	**0.62**	**0.070 - 5.56**	**0.67**
Maturity	**Subadult**	**1**		
	Adult	**0.58**	**0.27 - 1.28**	**0.18**
Sex	Female	1		
	Male	1.31	0.80 - 2.15	0.29
Active pouch[2]	No	1		
	Yes	1.80	0.66 - 4.93	0.25

[1] OR = odds ratio

[2] In adult females. Active pouch = lactating, not active pouch = not lactating

Table 8.2 Univariable risk factors for *Giardia* spp. infection in Perth quenda - concurrent ectoparasitic infections

Variables with an association considered strong enough to be included in multivariable analysis are shown in bold.

		Crude OR[1]	95% CI	p-value
Flea burden (Order Siphonaptera)	**Nil**	**1**		
	Low	**3.41**	**1.83 - 6.33**	**< 0.001**
	High	**5.00**	**2.48 - 10.09**	**< 0.001**
Mesostigmatan mite burden (Order Mesostigmata)	**Nil**	**1**		
	Low	**1.59**	**0.88 - 2.87**	**0.13**
	High	**2.14**	**1.16 - 3.94**	**0.014**
Trombiculid mites (Suborder Prostigmata)	Absent	1		
	Present	0.96	0.49 - 1.89	0.91
Tick burden (Family Ixodidae)	Nil	1		
	Low	1.02	0.59 - 1.75	0.95
	High	1.66	0.70 - 3.94	0.25

[1] OR = odds ratio

Table 8.3 Univariable risk factors for *Giardia* spp. infection in Perth quenda - concurrent gastrointestinal parasitic infections

Variables with an association considered strong enough to be included in multivariable analysis are shown in bold.

		Crude OR[1]	95% CI	p-value
***Cryptosporidium* spp.**	**Not infected**	**1**		
	Infected	**17.2**	**7.33 - 40.6**	**< 0.001**
***Labiobulura* spp.**	**Not infected**	**1**		
	Infected	**3.40**	**1.46 - 7.93**	**0.005**
***Linstowinema* spp.**	**Not infected**	**1**		
	Infected	**1.76**	**1.04 - 2.97**	**0.034**
***Trichuris* spp.**	**Not infected**	**1**		
	Infected	**1.53**	**0.94 - 2.50**	**0.090**
Coccidian oocysts[2] (Family Eimeriidae)	Not infected	1		
	Infected	1.50	0.47 - 4.84	0.50
Entamoeba spp.	Not infected	1		
	Infected	1.43	0.57 - 3.63	0.45
Potorolepis spp.	Not infected	1		
	Infected	0.93	0.44 - 1.94	0.84
Strongyles (Infraorder Rhabditomorphia)	Not infected	1		
	Infected	1.63	0.32 - 8.21	0.56
Strongyloids (Family Strongyloididae)	Not infected	1		
	Infected	0.92	0.46 - 1.85	0.82
Physaloptera spp.	Not infected	1		
	Infected	0.93	0.31 - 2.80	0.90

[1] OR = odds ratio

[2] Sporulation of coccidian oocysts was attempted on faecal samples from 71 quenda - all sporulated oocysts were *Eimeria* spp. (Chapter 10)

On multivariable analyses, only *Cryptosporidium* spp. infection and flea burden infection remained associated with the odds of *Giardia* spp. infection (Table 8.4). The independent variables maturity, *Trichuris* spp. infection, *Pygiopsylla* spp. infection, *Stephanocircus dasyuri* infection and mesostigmatan mite infection did not improve the multivariable model fit, and thus were omitted.

Table 8.4 Risk factors for *Giardia* spp. infection in Perth quenda - multivariable analysis[1]

		Adjusted OR[2]	95% CI
Trapping site	Bushland	1	
	Urbanised	1.45	0.75 - 2.81
Season	Autumn	1	
	Spring	1.70	0.84 - 3.44
	Winter	1.78	0.12 - 25.5
***Cryptosporidium* spp.**	**Not infected**	**1**	
	Infected	**17.4**	**6.95 - 43.6**
Labiobulura spp.	Not infected	1	
	Infected	2.61	0.98 - 6.96
Linstowinema spp.	Not infected	1	
	Infected	1.87	0.90 - 3.86
Flea burden	**Low**	**1**	
	Nil	**2.94**	**1.35 - 6.41**
	High	**3.66**	**1.50 - 8.94**

[1] The independent variables maturity, *Trichuris* spp. infection and mesostigmatan mite infection did not improve the multivariable model fit, and thus were not retained in the multivariable model.

[2] OR = odds ratio

8.3.3 Geographical clustering of *Giardia* spp. infection risk

Quenda infected with *Giardia* spp. were identified throughout the greater Perth region (Figure 8.1).
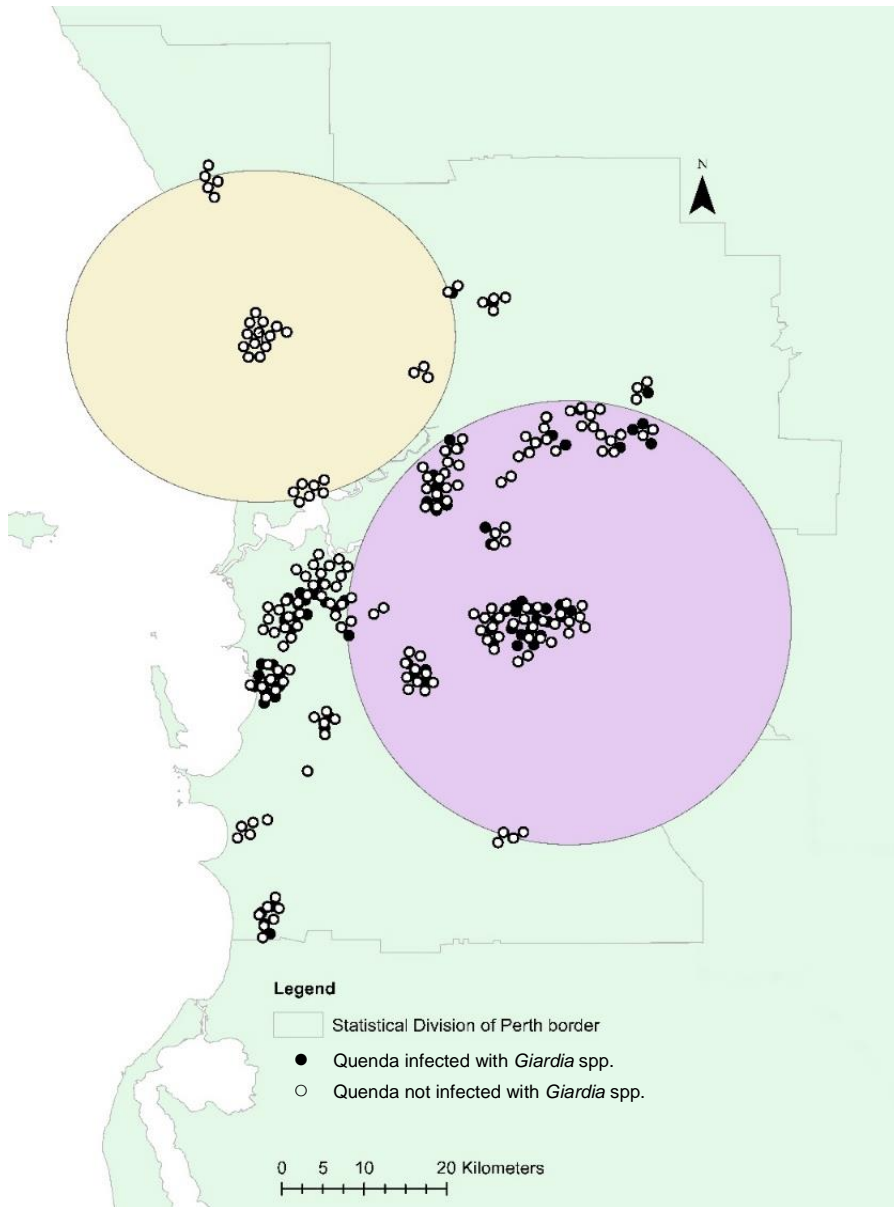

Figure 8.1 Geographical distribution of *Giardia* spp. infection in Perth quenda

Quenda were mapped using the GPS position of the location at which it was trapped. GPS points were jittered by 0.00001 decimal degrees, to facilitate visualisation of the relative distribution of *Giardia* spp. infection in the greater Perth region. By Kulldorff's spatial scan statistic, the beige circle (northwest) represents the most likely cluster of *Giardia* spp. infection in Perth quenda (OR of infection < 0.001, compared to quenda trapped elsewhere in Perth; $p < 0.001$). The purple circle (southeast) represents the second most likely cluster of *Giardia* spp. infection in Perth quenda (OR of infection 1.75, compared to quenda trapped elsewhere in Perth; $p = 0.35$).

This study found no evidence of an increase in *Giardia* spp. infection risk in quenda in urbanised environments compared to bushland (adjusted OR 1.45, 95% CI 0.75 – 2.81; Table 8.4). However, while the predominant *G. peramelis* was identified in quenda trapped in bushland and urbanised environments, *G. duodenalis* and *G. canis* genotype D were only identified in quenda trapped in urbanised environments.

On Kulldorff's spatial scan statistic, the most likely cluster of infection was identified in the north-west aspect of the greater Perth region. Quenda trapped in this area had < 0.001 times the odds of infection than quenda trapped elsewhere in the greater Perth region (p < 0.001). The second most likely cluster was identified in the south-east area of the greater Perth region. Quenda trapped in this area had 1.75 times the odds of infection than quenda trapped elsewhere in Perth (p = 0.35) (Figure 8.1).

8.3.4 Associations between *Giardia* spp. infection and indicators of ill health

Observations on faecal condition, skin condition and fur condition were available from all trapped quenda, while subjective body condition was missing from one animal. There was no evidence of an association between *Giardia* spp. infection and any of these variables (p > 0.10; Table 8.5).

Table 8.5 Association between *Giardia* spp. infection and indicators of ill-health in Perth quenda

		Crude OR[1]	95% CI	p-value
Faecal condition	Formed	1		
	Poorly formed	1.24	0.55 – 2.75	0.61
Subjective body condition	Optimal	1		
	Low condition	0.75	0.23 – 2.44	0.63
	High condition	1.38	0.82 – 2.33	0.23
Fur condition	Full coat	1		
	Incomplete coat	1.40	0.86 – 2.29	0.18
Skin condition	Normal	1		
	Scale	0.52	0.11 – 2.57	0.43

[1] OR = odds ratio

Forty-three out of 116 adult female quenda and 139 / 140 adult male quenda were included in testing the association between *Giardia* spp. infection and objective body condition. All excluded adult female quenda had pouch young, and the excluded adult male quenda did not have body weight recorded, which precluded calculation of their objective body condition scores. There was no evidence of an association between *Giardia* spp. infection and objective body condition in either adult female quenda (regression coefficient = -52.9, 95% CI -170.7 – 65.0; p = 0.37; adj. R^2 = -0.0043) or adult male quenda (regression coefficient = -10.8, 95% CI -97.2 – 75.6; p = 0.81; adj. R^2 = -0.0068).

8.4   Discussion

8.4.1   *Giardia* spp. prevalence

Giardia spp. infection was highly prevalent in Perth quenda, in both bushland and urbanised environments - although we cannot rule out bias to these prevalence estimates, as a result of the use of non-proportionate sampling, which was utilised out of practical necessity. Our study indicates that *Giardia* spp. infections of Perth quenda are typically the host-adapted, non-zoonotic *G. peramelis*. *Giardia duodenalis* and *G. canis* genotype D were rarely identified, and only in quenda trapped in urbanised environments. Thompson et al. (2009) suggest that domestic animals and / or humans, or dogs (respectively) are likely sources of these infections to quenda.

The apparent prevalence estimate of *Giardia* spp. infection is likely to closely reflect true prevalence of infection in our study sample, as immunofluorescence microscopy has been shown to be an accurate indicator of *Giardia* spp. infection in quenda (Chapter 4). The prevalences of the particular *Giardia* species are likely to have been underestimated, as not all infections successfully amplified on PCR, and some that amplified could not be sequenced (the PCR and sequencing protocol employed has been demonstrated to lack sensitivity in the detection of *Giardia* spp. infection in

quenda (Chapter 4)). However, it is unlikely that the success of PCR and sequencing would have been differentially biased based on the species of *Giardia* present, and we therefore believe the proportionate representation of the *Giardia* species in the prevalence estimates reflects that in Perth quenda.

This study provides the first documentation of *G. canis* genotype D infection in quenda.

8.4.2 *Giardia* spp. in quenda - risk factors for infection

The increased odds of *Giardia* spp. infection in quenda concurrently infected with *Cryptosporidium* spp., and concurrently infected with low and high flea burdens, are considered likely to be confounded by population density. Quenda in areas of higher population density may be more likely to be infected with *Cryptosporidium* spp., as well as having an increased odds of *Giardia* spp. infection, compared to quenda in areas of lower population density. Similarly, quenda trapped in areas of high population density may be more likely to be infected with fleas, and if infected with fleas have a greater intensity of infection, as well as increased odds of *Giardia* spp. infection, compared to quenda in areas of lower population density. We were unable to accurately measure population density as part of this survey, and thus account for it in our multivariable statistical analyses, as safety concerns for the animals in trapping in urbanised environments and bushland accessible to humans precluded the rigorous trapping layouts appropriate for measuring population density. However, subjectively, it seemed that quenda population density was consistently high to very high at urbanised sites in which quenda were trapped, whereas quenda population density appeared to vary widely in bushland where quenda were trapped.

We found no evidence that quenda in urbanised environments in Perth had an increased odds of *Giardia* spp. infection, beyond that which may be attributable to relatively increased population density. In the multivariable analysis, where infections with *Cryptosporidium* spp., *Acedestia chera*, *Labiobulura* spp. and *Linstowinema* spp.

were controlled for, urbanisation and *Giardia* spp. infections were no longer associated. This finding suggests that certain parasitic infections in quenda may provide population density proxies, in circumstances where population density cannot be measured.

8.4.3 Geographical clustering of *Giardia* spp. infection risk

The area of substantially decreased odds of *Giardia* spp. infection in quenda in north-west Perth and the area of increased odds of *Giardia* spp. infection in quenda in south-east Perth, identified on Kulldorff's spatial scan statistic, correlates with the best available evidence (Howard et al., 2014) and our own subjective observations of the areas of Perth with low and relatively high population density of quenda, respectively. The lack of statistical support for the cluster of increased infection risk may be attributable to a lack of power, given the relatively small sample size. The cluster of low infection risk may have been artificially strengthened by the edge effects of the coastline.

Giardia duodenalis and *G. canis* genotype D were only found in quenda trapped in urbanised environments. It is considered likely that quenda in urbanised environments are at increased risk of acquiring these species of *Giardia* than quenda in bushland, due to increased exposure to sources of infection, but this was not possible to test statistically in this study, given the infections' apparent rarity combined with this relatively small sample size.

8.4.4 Association between *Giardia* spp. infection and indicators of ill health

There was no evidence of an association between *Giardia* spp. infection and indicators of ill health such as faecal and body condition. However, these results should be considered representative of *G. peramelis* only, as infections with other *Giardia* spp. were too rare to have impacted study findings. The lack of temporal sequence in these

observations is a substantial limitation, so the lack of an association with ill health should be treated cautiously at this stage.

8.5   Conclusions

This study found no evidence that quenda in Perth are a substantial reservoir of zoonotic *Giardia* spp. infections, or of species of *Giardia* that are known to be infectious to domestic animals. Although some significant risk factors were identified on multivariable analysis, these are likely to have been confounded by population density and density is subsequently hypothesised to be a principal driver of infection risk. Although there was some evidence of spatial structuring of infection, this was not related to gradients of urbanisation and probably also reflected differences in quenda population density. There was no association between *Giardia* spp. infection and the indicators of ill health assessed in this study, but this is representative of *G. peramelis* infection only.

8.6   Acknowledgements

8.7 References

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Chapter 9

Confirmation of a unique species of *Giardia*, parasitic in the quenda (*Isoodon obesulus*)

This chapter is a published paper:

Author contributions:

Designed the study: AH, RCAT

Organised and undertook data collection: AH

Performed the immunofluorescence microscopy: AH

Performed the light microscopy: AH, AE

Performed PCR, sequencing and phylogenetic analyses: AA

Performed excystation: CP

Wrote and revised the manuscript: AH, AA, CP

Proofed and critically appraised the manuscript: AE, AL, RCAT

9. Confirmation of a unique species of *Giardia*, parasitic in the quenda (*Isoodon obesulus*)

9.1 Introduction

A novel genotype of *Giardia* was isolated and described from a quenda (syn. southern brown bandicoot, *Isoodon obesulus*) in southwest Western Australia (Adams et al., 2004). This was believed to be a distinct species of *Giardia*, but the lack of samples from additional infected animals precluded formal description of this genotype as a separate species at that time.

Since this initial description, the 'quenda genotype' of *Giardia* has been documented in quenda in several other locations in Western Australia (Thompson et al., 2010). It has also been identified using PCR from a calf in Western Australia, though it is unclear whether this reflected infection or cysts passing through the calf gut after ingestion of contaminated pasture (Ng et al., 2011a). The 'quenda genotype' of *Giardia* has not been isolated from other Australian marsupial species surveyed for *Giardia* spp. (McCarthy et al., 2008, Thompson et al., 2008, Thompson et al., 2010, Ng et al., 2011b, Thomasz, 2014, Vermeulen et al., 2015)

We undertook a parasitological survey of quenda in the greater Perth region, Western Australia, investigating the epidemiology of *Giardia* spp. infections in this species, and found infection with the 'quenda genotype' of *Giardia* to be common. We aimed to formally describe the 'quenda genotype' of *Giardia* as a separate species, *G. peramelis,* by describing the morphology of cysts and trophozoites and genetically characterising the parasite. It is recognised that for any parasite, once adequate data are available the names should be formalised at the species level (Brooks and Hoberg, 2000). This provides stability and is essential for effective communication. More specifically, we considered formal description of this parasite important to expand knowledge of the phylogenetic range of the genus *Giardia*; and in consideration of the

public health significance of *Giardia* spp. in Australian marsupials, in differentiating zoonotic and non-zoonotic 'strains' of the parasite.

9.2 Methods

9.2.1 Obtaining *Giardia peramelis* specimens

Faecal samples were collected from quenda trapped across 51 locations in the Statistical Division of Perth, Western Australia. Faecal material was also collected from the large intestine of quenda carcasses, obtained opportunistically from the same area. All samples were obtained under Murdoch University Animal Ethics Permit (R2530/12), and Department of Parks and Wildlife Regulation 17 (SF009640) and Regulation 4 (CE004287) permits.

From each quenda, 2 mL faeces were thoroughly mixed in to 8 mL 10% buffered formalin, and 1 mL faeces were thoroughly mixed in to 8 mL 70% ethanol. Preserved faecal samples were stored at 4°C until analysis.

The formalin-preserved faecal samples were screened for *Giardia* spp. cysts using immunofluorescence microscopy. Merifluor Cryptosporidium / Giardia kits (Meridian Bioscience, Inc. USA) were used according to manufacturer's directions for unconcentrated faecal samples. Slides were examined at 200x magnification. Samples positive for *Giardia* spp. were differentiated to a species level via PCR and sequencing (methodology in section 9.2.4). In addition, ten immunofluorescence negative samples from trapped quenda were randomly selected and subject to the same PCR and sequencing protocols as the immunofluorescence positive samples.

9.2.2 *Giardia peramelis* morphological description - trophozoites

Wet mounts were prepared from the small intestinal mucosa of two quenda carcasses, which were positive for *G. peramelis* on faecal testing (and were not positive for any

other species of *Giardia*), and were considered sufficiently fresh, with minimal mucosal autolysis, for detection of trophozoites. The first third of the small intestine was gently scraped and the scrapings mounted on a microscope slide. The slides were examined for trophozoites using an Olympus BX50 microscope. A sample of mucosal scrapings from each quenda was also used to seed flasks containing *Giardia* media (section 9.2.2, below), and cultures were monitored regularly for the appearance of trophozoites.

Excystation of *G. peramelis* cysts was attempted three times, using faecal samples from three quenda that were positive for *G. peramelis* by immunofluorescence microscopy and PCR (and not positive for any other species of *Giardia*).

To purify *G. peramelis* cysts, 1 gram of fresh faeces containing *G. peramelis* was homogenised in 1X PBS, passed through layers of gauze, and centrifuged at 0.6 G. Two wash steps were carried out, where the supernatant was removed, the pellet was resuspended in 1X PBS, and the sample was centrifuged at 0.6 G. Two sucrose samples were made - one to a specific gravity (SG) of 1.18, and another made up to 0.8 M. The 0.8 M solution was layered on top of the SG 1.18 solution, and the purified sample was layered on top. The sample was centrifuged at 0.2 G, and cysts were collected at the water / sucrose interphase. 1X PBS was added to this isolation and it was centrifuged at 0.6 G, with the supernatant removed subsequently. The cysts were resuspended in a final volume of 1 mL 1X PBS, and examined under the microscope.

For excystment, cysts were resuspended in 10 mL of acidified Hanks Balanced Salt Solution (HBSS) (30 g / L biosate peptone (BD, Annapolis, USA), 10 g / L glucose (Sigma-Aldrich, St. Louis, USA), 2 g / L sodium chloride (Chem-Supply, Adelaide, Australia), 2 g / L cysteine (Sigma-Aldrich, St. Louis, USA), 1 g / L K_2HPO_4 (Chem-Supply, Adelaide, Australia), 0.6 g / L KH_2PO_4 (Merck, Melbourne, Australia), 0.01 g / L ferric ammonium citrate (Sigma-Aldrich, St. Louis, USA), 0.2 g / L ascorbic acid

(Sigma-Aldrich, St. Louis, USA), 0.5 g / L bovine bile (Sigma-Aldrich, St. Louis, USA), 100 mL / L newborn calf serum (SAFC Biosciences, Lenexa, USA) and 10 mL / L penicillin / streptomycin (Sigma-Aldrich, St. Louis, USA), pH 2) and incubated at 37°C for 30 minutes. Cysts were then centrifuged at 0.2 g and washed twice in HBSS (pH7.2), and finally resuspended in HBSS (pH 7.2) medium and incubated at 37°C. Cysts were monitored daily for excystment, for one week.

9.2.3 *Giardia peramelis* morphological description - cysts

To describe the morphology of *G. peramelis* cysts, faecal smears were prepared using formalin-preserved faecal samples from eight quenda that were positive for *G. peramelis* by PCR with sequencing (and not positive for any other species of *Giardia*). Smears were examined by bright field and Nomarski differential interference microscopy, using an Olympus BX50 microscope. *Giardia peramelis* cysts were photographed at 1000x magnification. ImageJ software (US National Institute of Health, Bethesda, Maryland), was used to measure cyst length and width.

9.2.4 *Giardia peramelis* molecular characterisation

9.2.4.1 DNA extraction and PCR amplification

Amplification by PCR was attempted on all immunofluorescence microscopy positive faecal samples and the ten randomly selected immunofluorescence negative samples. Ethanol-preserved faecal samples were centrifuged to separate ethanol from faeces, with the ethanol supernatant discarded. Samples were then twice re-homogenised in distilled water, centrifuged and supernatant discarded. DNA extraction was then conducted using the Maxwell® 16 Instrument (Promega, Madison, USA) as per manufacturer's instruction.

Amplification by PCR was attempted at three loci: 18S rRNA, ITS1-5.8s-ITS2 and glutamate dehydrogenase (*gdh*). Initially, a semi-nested PCR protocol was employed to amplify a 130 bp product of the 18s rRNA, with primers RH11/RH4 and RH11/GiarR

(Hopkins et al., 1997, Read et al., 2002). The PCR reaction was performed in 25 µL volumes, consisting of 1-2 µL of extracted DNA, 2.0 mM $MgCl_2$, 1 x reaction buffer (20 mM Tris-HCL, pH 8.5 at 25°C, 50 mM KCl), 400 µM of each dNTP, 0.4 µM of each primer, 0.5 units of Taq DNA polymerase (Fisher Biotec, Perth, Australia), and DMSO 5%. Amplification conditions were modified from Hopkins et al. (1997), and involved a denaturing step of 95°C for 6 minutes, then 40 cycles of 95°C for 30 seconds, 53°C for 30 seconds (56°C in the secondary round) and 72°C for 30 seconds, followed by a final extension of 72°C for 7 minutes.

A nested PCR protocol was conducted to amplify a 330 bp product of the ITS1-5.8S-ITS2 region of the ribosomal gene, with primers developed by Caccio et al. (2010). The PCR reactions were the same as those used for 18s rRNA, but performed in 50 µL volumes. Conditions for amplifications were modified from Caccio et al. (2010), and involved an initial denaturing step of 95°C for 5 minutes, then 40 cycles of 95°C for 45 seconds, 59°C for 30 seconds and 72°C for 30 seconds, followed by a final extension of 72°C for 7 minutes.

Finally, for *gdh*, a nested PCR protocol was used to amplify a 530 bp product, using the primer pairs Gdh1/Gdh2 and Gdh3/Gdh4 for the primary and secondary rounds respectively, as per Caccio and Ryan (2008). The PCR reaction was performed in 25 µL volumes, consisting of 2 µL of extracted DNA, 1.5 mM $MgCl_2$, 1 x reaction buffer, 200 µM of each dNTP, 0.4 µM of each primer, 1 unit of Taq DNA polymerase (Fisher Biotec, Perth, Australia) and DMSO 5%. Conditions for amplifications were the same for both rounds, and involved an initial denaturing step of 94°C for 2 minutes, then 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 60 seconds, followed by a final extension of 72°C for 7 minutes.

9.2.4.2   Sequencing of amplified product

PCR products were purified using an Agencourt AMPure XP system (Beckman coulter, Beverly, USA). Sequence reactions were performed using the Big Dye Terminator Version 3.1 cycle sequencing kit (Applied Biosystems), according to the manufacturer's instructions. Reactions were electrophoresed on an ABI 3730 48 capillary machine. Amplicons were sequenced in both directions, with resultant nucleotide sequences compared with published sequences on NCBI GenBank® using the basic alignment search tool (BLAST). Further sequence analysis was conducted using the sequence alignment program Sequencer™ 4.8 (Gene Codes, Ann Arbor, MI, USA).

Nucleotide sequence data reported in this paper are available in the NCBI GenBank® database under accession numbers KU306911, KU306912, KU306913, KU306914 and KU306915.

9.2.4.3   Phylogenetic analyses

Phylogenetic analyses of sequences obtained in this study were conducted using the programme MEGA6 (Tamura et al., 2013). Phylogenetic trees were inferred with the neighbour-joining method, with a bootstrapping of 1000 replicates. In addition, analyses were conducted using the maximum likelihood and maximum parsimony methods. Evolutionary distances were calculated using the Kimura 2-parameter method (Kimura, 1980). Published sequences representing *G. muris*, *G. ardeae*, *G. microti* and all assemblages within the '*G. duodenalis* species complex' (*G. duodenalis, G. enterica, G. canis, G. bovis, G. cati* and *G. simondi*) were retrieved from GenBank® (accession numbers for 18S rRNA: X65063, Z17120, AF006676, X52949, AF199446, DQ100287, AF199447, U09491, AF199443, AF199449, AF199448, AF199444, AF199450 and AY309064; accession numbers for ITS1-5.8s-ITS2: GU126450, X65063, X58290, M73684, GU124448, X52949, GU126432/33/34/35, AF239840, U09491, GU126437/38/40/43/44/45).

9.3 Results

Faecal samples positive for *Giardia* spp. by immunofluorescence microscopy were obtained from 99 trapped quenda, and 11 quenda carcasses. Of the immunofluorescence positive samples, 63 / 99 trapped quenda and 11 / 11 quenda carcasses were confirmed to be infected with *G. peramelis* by PCR and sequencing at one or more loci. Thirty-six immunofluorescence positive faecal samples from trapped animals either did not amplify by PCR, or amplified product failed to give a readable sequence.

Of the ten randomly selected immunofluorescence microscopy negative samples tested by PCR and sequencing, one was positive for *G. peramelis* by PCR and sequencing at one locus.

9.3.1 *Giardia peramelis* morphology

Giardia peramelis trophozoites were not detected in intestinal scrapings of the two tested quenda carcasses, or in the cultures seeded with mucosal scrapings from the two quenda, and all attempts at *G. peramelis* excystation failed. This precluded morphological description of *G. peramelis* trophozoites.

Giardia peramelis cysts are morphologically indistinguishable from cysts of the '*G. duodenalis* species complex' and *G. microti* (Figures 9.1 and 9.2). *Giardia peramelis* cysts had an average length of 12.68 µm (standard deviation 0.72 µm; range 11.07 – 14.15 µm). Cyst width averaged 7.88 µm (standard deviation 0.47 µm; range 6.78 – 9.03 µm).
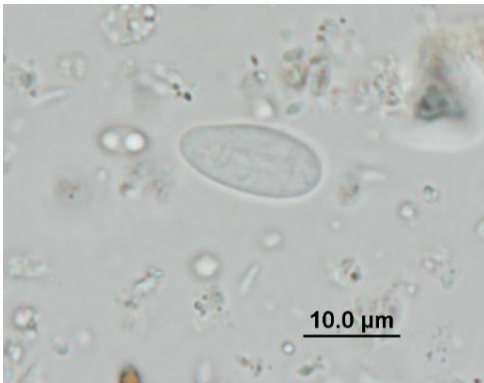
Figure 9.1 Cyst of *Giardia peramelis* by light microscopy
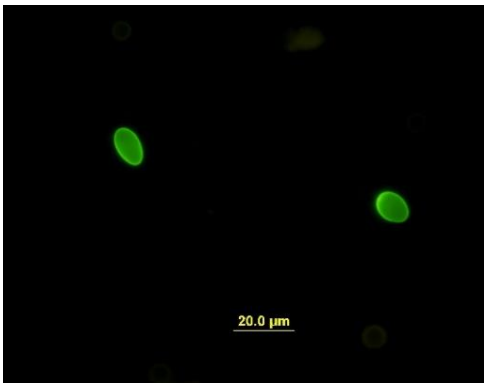
Figure 9.2 Cysts of *Giardia peramelis* by immunofluorescence microscopy

9.3.2   *Giardia peramelis* molecular characterisation

Of the 111 individual quenda faecal samples found positive for *Giardia* spp. by

immunofluorescence microscopy or PCR, 75 (67.6%) were successfully amplified and

sequenced at one or more loci. Sixty-four samples were sequenced at the 18s rRNA

locus, 50 samples sequenced at ITS1-5.8s-ITS2 and two samples sequenced at the

gdh locus (Table 9.1). Two of the 75 samples sequenced at all three loci, 37 samples

sequenced at both 18S and ITS1-5.8s-ITS2, and the remaining 36 samples sequenced

at only one locus (Table 9.2).

Table 9.1 Molecular characterisation results obtained at three loci for each quenda faecal sample positive for *Giardia* spp. by immunofluorescence microscopy or PCR and sequencing (n = 111)

Sample source (n)	Number of *Giardia* spp. positive quenda samples successfully amplified and sequenced at each locus:		
	18S rRNA	ITS1-5.8s-ITS2	*gdh*
Trapped, IMF[1] +ve quenda (n = 99)	54	42	2
Trapped, IMF[1] –ve quenda (n = 1)	0	1	0
Quenda carcasses (n = 11)	10	7	0
Total no. successfully characterised at locus / total *Giardia* spp. positives	**64 / 111 (57.7%)**	**50 / 111 (45.0%)**	**2 / 111 (1.8%)**

[1] IMF = immunofluorescence microscopy

Table 9.2 Combined molecular characterisation results for quenda faecal samples confirmed to be positive for *G. peramelis* on PCR and sequencing (n = 75)

Sample source (n)	Loci at which samples were successfully amplified and sequenced:				
	18S only	ITS only	*gdh* only	18S & ITS only	18S & ITS & *gdh*
Trapped, IMF[1] +ve quenda (n = 63)	21	9	0	31	2
Trapped, IMF[1] –ve quenda (n = 1)	0	1	0	0	0
Quenda carcasses (n = 11)	4	1	0	6	0
No. characterised at locus combination/ total *G. peramelis* positive quenda	**25 / 75 (33.3%)**	**11 / 75 (14.7%)**	**0 / 75 (0%)**	**37 / 75 (49.3%)**	**2 / 75 (2.7%)**

[1] IMF = immunofluorescence microscopy

Of the 116 sequences obtained, 114 were the novel *G. peramelis*; two sequences obtained at the 18S rRNA locus were suspected to be mixed infections of *G. duodenalis* / *G. peramelis* and *G. canis* / *G. peramelis*. These 'mixed infection' samples were also amplified at the ITS1-5.8s-ITS2 locus - the resultant sequences were clearly identified as *G. peramelis*, with no ambiguous nucleotides evident.

Utilizing the BLAST tool within NCBI, the 18S rRNA sequences were highly similar to the published sequence AY309064, previously reported as the 'quenda genotype'. Alignment in Sequencer™ revealed one single nucleotide polymorphism (SNP) between sequences obtained in this study (represented by QBY95 and QM22) and AY309064. In addition, one SNP was identified within a small subset of sequences obtained in this study, represented by sequence QBN13. A large region of extreme variability was identified between all sequences and another reported 'quenda genotype'. Of the 292 bp published for this sequence (accession number HQ398319), a region of approximately 150 bp showed extreme mismatch.

Sequences obtained at ITS1-5.8S-ITS2 required the BLAST program selection of 'somewhat similar sequences' in order to achieve a result. The '*G. duodenalis* species complex' was the primary match, but achieved low coverage and identities with all comparisons. Alignment of these sequences in Sequencer™ revealed one SNP, represented by samples QBY95 / QM22 and QBN13. Similar BLAST results were obtained with the two *gdh* sequences.

9.3.3 Phylogenetic analysis

Very similar trees were obtained by neighbour-joining, maximum likelihood and maximum parsimony methods; only the neighbour-joining trees are presented here. Phylogenetic analysis of sequence data obtained at 18s rRNA confirmed that the *Giardia* genotype obtained from quenda in this study (*G. peramelis*) formed a separate clade with the 'quenda genotype' reference AY309064, and was distinct from all assemblages within the '*G. duodenalis* species complex' and *G. microti* (Figure 9.3).
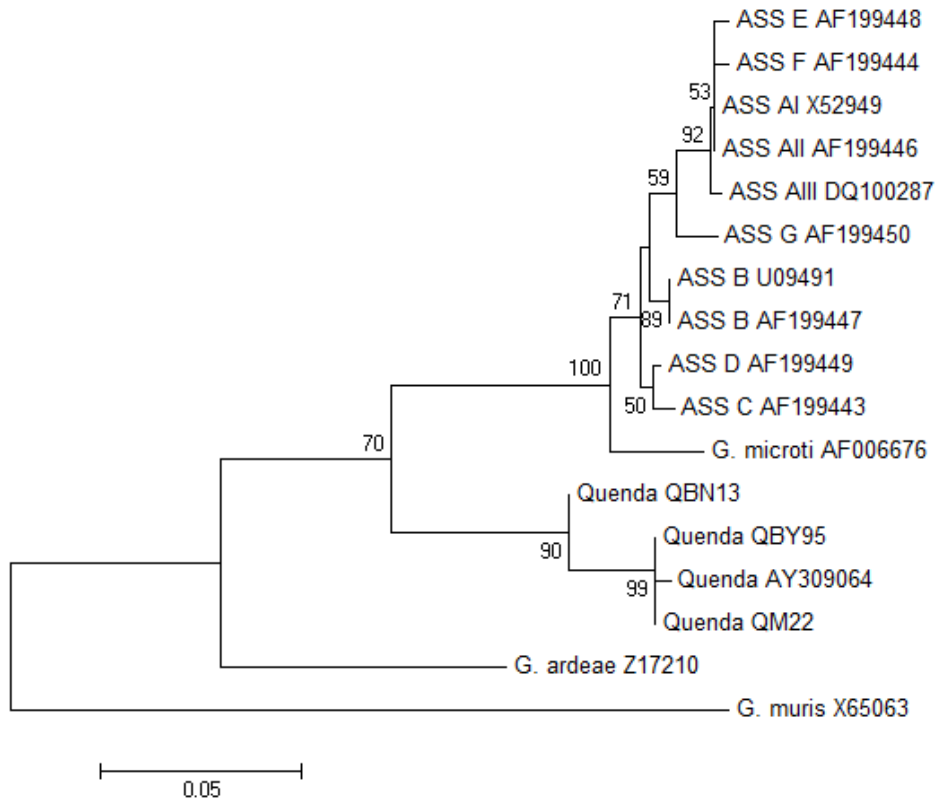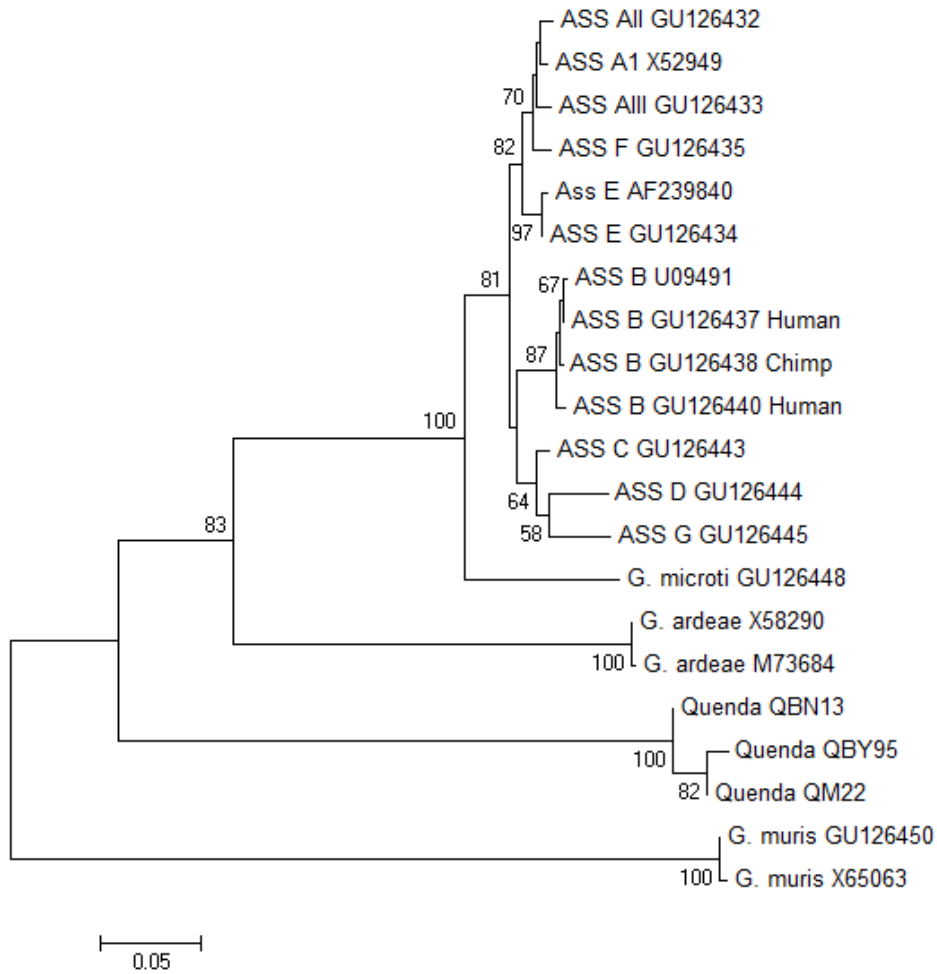
Figure 9.3 Phylogenetic relationships of *Giardia peramelis* isolates obtained in this study (quenda QBN13, QM22, QBY95) with published reference material available at the 18s rRNA locus

Evolutionary history inferred using the neighbour-joining method supported with bootstrap test of 1000 replicates (values > 50% shown). *G. muris* is used as the out group.

A similar topology was observed based on genetic data obtained at ITS1-5.8S-ITS2, with the exception that *G. peramelis* was also placed external to *G. ardeae* (Figure 9.4). Phylogenetic analysis of *gdh* also placed the *G. peramelis* external to all assemblages within the 'G. duodenalis species complex' (results not shown).

Figure 9.4 Phylogenetic relationships of *Giardia peramelis* isolates obtained in this study (quenda QBN13, QM22, QBY95) with published reference material available at the ITS1-5.8S-ITS2 locus.

Evolutionary history inferred using the neighbour-joining method supported with bootstrap test of 1000 replicates (values > 50% shown). *G. muris* is used as the out group.

9.4 Discussion

9.4.1 *Giardia peramelis* taxonomic summary

Type host: *Isoodon obesulus* - quenda (syn. southern brown bandicoot)

Mammalian additional hosts: unknown

Type locality: Perth, Western Australia

Additional locations: other locations in Western Australia (Adams et al., 2004; Thompson et al., 2010).

Site of Infection: unknown; presumably small intestine, based on the known *Giardia* spp. site of infection in other mammalian hosts (Monis et al., 2009)

Prepatent and patent periods: unknown

Material deposited: A sample of formalin-preserved and ethanol-preserved *G. peramelis* cysts, and photomicrographs of *G. peramelis* cysts, were deposited at the Western Australian Museum (specimen registration no. WAM Z68785)

Etymology: The specific epithet *peramelis* is derived from Subfamily Peramelinae / Family Peramelidae / Order Peramelemorphia - taxonomic classifications of the quenda, the only confirmed host of *G. peramelis*. This is in line with current taxonomic nomenclature for *Giardia* spp. (Monis et al., 2009).

This study confirms that *G. peramelis* (previously the 'quenda genotype' of *Giardia*) is a unique species. Based on genotyping at 18s rRNA, all *G. peramelis* isolates in this study strongly aligned with the original 'quenda genotype' data, generated by Adams et al. (2004). We have further confirmed the genetic novelty of *G. peramelis* with genetic data obtained at ITS1-5.8S-ITS2, which identified *G. peramelis* as belonging to the genus *Giardia* but did not match any published sequences of *Giardia* spp. Phylogenetic analyses of both genes place *G. peramelis* external to the '*G. duodenalis* species complex' and external to *G. microti*. This supports the proposals of both Adams et al. (2004) and Thompson and Monis (2004, 2012) that *G. peramelis* is a novel lineage, distinct from described species.

The extreme mismatch observed between the *G. peramelis* 18S rRNA sequences reported here and in Adams et al. (2004), and the previously reported 'quenda genotype' (HQ398319) obtained from a calf (Ng et al., 2011a), suggests that a partial 'quenda genotype' may have been sequenced from the calf, along with other genetic material.

As *G. peramelis* cysts are morphologically indistinguishable from several other species of *Giardia*, molecular characterisation is required to differentiate *G. peramelis* from other *Giardia* spp. Our results suggest that the 18S rRNA and ITS1-5.8s-ITS2 loci are the most successful target regions for this purpose.

The substantial number of quenda observed to be infected with *G. peramelis*, sampled across a large number of locations in this study, suggests that quenda are a natural host for *G. peramelis*.

9.5   Conclusions

This study confirms that *G. peramelis* (formerly the 'quenda genotype' of *Giardia)* is a unique species of *Giardia*. It expands on known genetic and morphological data of this species, and describes techniques used in the genetic characterisation of *G. peramelis*.

9.6　　Acknowledgements

9.7 References

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Chapter 10

Eimeria spp. infecting quenda (*Isoodon obesulus*) in the greater Perth region, Western Australia

This chapter is intended for publication.

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Author contributions:

Designed the study: AEH, RCAT

Organised and undertook data collection: AEH

Undertook sporulation and morphological description of Eimeria angustus: AEH

Undertook Eimeria spp. PCR, sequencing and phylogenetic analyses: RY

Wrote and revised the manuscript: AEH

Proofed and critically appraised the manuscript: RY, AJL, RCAT

10. *Eimeria* spp. infecting quenda (*Isoodon obesulus*) in the greater Perth region, Western Australia

10.1   Introduction

Parasites of wildlife inhabiting urbanised or peri-urbanised environments are of importance because of their potential impact on population health (Thompson et al., 2009). The role of parasites as a density-dependent regulator of host population size may be of particular significance in this case, as urbanisation can be associated with abnormally increased population densities of wildlife species that adapt to living in urban areas (Bradley and Altizer, 2007). Additionally, zoonotic parasites present in wildlife in urban or peri-urban areas are of public health significance (Mackenstedt et al., 2015).

Though urbanisation is associated with habitat loss for wildlife in Australia and worldwide (McKinney, 2002; Garden et al., 2006), some wildlife species are able to adapt and survive in urbanised environments. Quenda (syn. southern brown bandicoots, *Isoodon obesulus*) are a small, terrestrial peramelid marsupial, and have survived in many urbanised areas of Perth, Western Australia (Howard et al., 2014). Published documentation of parasites infecting quenda in this region are restricted to small samples of quenda tested for *Giardia* spp. (Thompson et al., 2010) and *Eimeria* spp. (Bennett and Hobbs, 2011).

Eimeria is a genus of apicomplexan parasites, species of which have been recorded in a wide range of vertebrates (including mammals, birds, reptiles and fish). Two species of *Eimeria* have been documented parasitising peramelid marsupial hosts: *E. kanyana*, parasitic in western barred bandicoots (*Perameles bougainville*) (Bennett et al., 2006), and *E. quenda*, parasitic in quenda (Bennett and Hobbs, 2011). *Eimeria* spp. have also been described, morphologically and in some cases genetically, from a range of other Australian marsupial species (Mykytowycz, 1964; Barker et al., 1988a,b,c; Barker et al.,

1989; O'Callaghan and O'Donoghue, 2001; Power et al., 2009; Hill et al., 2012; Austen et al., 2014). Although *Eimeria* species are typically host-specific (Joyner, 1982), there are a number of examples of infection of multiple host species within the same marsupial genus (*Macropus* spp.- Barker et al., 1988a; Barker et al., 1989; *Trichosurus* spp.- O'Callaghan and O'Donoghue, 2001; Power et al., 2009).

Eimeria spp. are an important cause of gastrointestinal illness in livestock (Chapman et al., 2013). However, though morbidity and mortality attributable to coccidiosis is known to occur in various Australian marsupial species (e.g. Winter, 1959; Barker et al., 1972), no studies have specifically investigated how commonly morbidity and mortality occur following *Eimeria* spp. infection in these hosts. Previous research has suggested that the pathogenicity of various *Eimeria* spp. in certain marsupial hosts may be mild in otherwise healthy individuals (Mykytowycz, 1964; Bennett et al., 2006).

This study aimed to:

1) identify, and estimate the prevalence of, species of *Eimeria* present in quenda;

2) morphologically describe and genetically characterise a novel observed species of *Eimeria* as *E. angustus;* and

3) genetically characterise the previously described species *E. kanyana*, for the purpose of distinguishing it genetically from *E. angustus*.

10.2 Methods

The target host population was free-ranging quenda in the Statistical Division of Perth. Quenda were trapped using Sheffield (cage) traps. Trapping was undertaken on 29 bushland sites and 35 urbanised sites (7 private non-residential properties and 28 private residential properties), from March 2013 to July 2015 (Chapter 7). A subset of trapped quenda were included in this study. Inclusion was based on the availability of fresh faeces from the animal. Trapping and sampling was undertaken under Murdoch University Animal Ethics Permit (R2530/12), and Department of Parks and Wildlife Regulation 17 (SF009640) and Regulation 4 (CE004287) permits.

10.2.1 Identifying species of *Eimeria* present in Perth quenda

Faeces were collected directly from the traps after removal of the animal, and traps were cleaned and disinfected between animals. Faecal samples may have been passed from the animal up to approximately 10 hours before collection from the trap. Faecal samples were stored in an insulated field box until processed, which was no later than 6 hours after collection.

One mL of faeces was preserved for genetic characterisation by mixing thoroughly into 4 mL 70% ethanol. Preserved samples were stored at 4˚C until analysis. The rest of the fresh faeces was mixed 1:4 into 2% potassium dichromate solution ($K_2Cr_2O_7$). This faecal mixture was poured into small petri dishes, and left in a dark cupboard at room temperature to sporulate. The faecal mixture was checked intermittently for sporulation, by concentrating oocysts present in a small portion of the sample by zinc sulphate flotation, and examining the sample microscopically. Briefly, a portion of faecal mixture was centrifuged at 850 G for 2 minutes, with supernatant discarded, and then resuspended in distilled water and re-centrifuged twice, to remove potassium dichromate. Samples were then mixed thoroughly with zinc sulphate solution (SG 1.18)

at a ratio of 1:4, and centrifuged at 850 G for 2 minutes. A flamed wire loop was used to transfer surface material (containing coccidian oocysts) on to a slide. Sporulated oocysts were examined at 400x to 1000x magnification, using an Olympus BX50 microscope. Photographs of sporulated oocysts were taken using bright field and Nomarski differential interference microscopy.

10.2.2 Prevalence estimates

Only oocysts that sporulated and could be unequivocally assigned to species based on morphology were included in estimates of prevalence. Ninety-five per cent confidence intervals were calculated using Jeffrey's method (Brown et al., 2001).

10.2.3 Morphological description of *Eimeria angustus*

One hundred and fifteen sporulated *Eimeria* spp. oocysts of a consistent, novel morphology (obtained from 21 quenda hosts) were examined and photographed at 1000x magnification, using bright field and Nomarski differential interference microscopy (Olympus BX50 microscope). Images were analysed using ImageJ software (US National Institute of Health, Bethesda, Maryland), to obtain measurements of oocyst length and width, oocyst wall thickness and sporocyst length and width. Due to the compacted nature of this species of *Eimeria,* measurements were only taken from one sporocyst per oocyst - the sporocyst that was subjectively identified as being positioned laterally. Where no sporocysts could be manipulated into lateral position within the oocyst, sporocyst length measurement was not taken.

10.2.4 Genetic characterisation of *Eimeria kanyana* and *Eimeria angustus*

Ethanol-preserved faecal samples from two quenda were used to characterise *E. kanyana*, and ethanol-preserved faecal samples from five quenda were used to characterise *E. angustus*, at the nuclear 18S rRNA and mitochondrial cytochrome

oxidase (COI) loci. *Eimeria quenda* was not characterised as part of this study, due to logistical limitations.

DNA was extracted using the Power Soil DNA Kit (MolBio, Carlsbad, California), as described in Yang et al. (2016). A nested PCR protocol was employed to amplify a 1285 bp product of the 18s rRNA locus, using methods described in Yang et al. (2016). A nested PCR protocol was also used to amplify sequences in subunit I of the COI gene, using methods described in Dolnik et al. (2009). Amplified products were purified, and sequenced using forward and reverse primers, as described in Yang et al. (2016).

Phylogenetic analyses of sequences were undertaken using MEGA6 (Tamura et al., 2013). Maximum likelihood, neighbour-joining and maximum parsimony analyses were all conducted, using additional isolates retrieved from GenBank®. These additional isolates comprised published sequences representing all possible host families (Tables 10.1 and 10.2). The robustness of nodes within the resulting trees were inferred from 1000 cycles of bootstrap resampling.

Table 10.1 18s rRNA sequences used in the *Eimeria* spp. phylogenetic analyses

Sequence no.	Species	Host and geographical origin
JQ993660	*Eimeria* sp.	*Apodemus agrarius* (striped field mouse) - Czech Republic
JQ993649	*Eimeria caviae*	*Cavia porcellus* (guinea pig) - Czech Republic
JQ993650	*Eimeria chinchillae*	*Chinchilla lanigera* (long-tailed chinchilla) - Czech Republic
AF324216	*Eimeria scholtysecki*	*Dipodomys agilis* (agile kangaroo rat) - USA
JQ993664	*Eimeria* sp.	*Gerbillus dasyurus* (Wagner's gerbil) - Jordon
AB769556	*Eimeria alabamensis*	*Bos taurus* (cattle) - Japan
AB769601	*Eimeria bukidnonensis*	*Bos taurus* (cattle) - Japan
HG793041	*Eimeria dispersa*	*Meleagris gallopavo* (turkey) - Europe
HG793045	*Eimeria innocua*	*Meleagris gallopavo* (turkey) - Europe
JQ993667	*Eimeria* sp.	*Mastomys natalensis* (natal multimammate mouse) - Malawi
JQ993661	*Eimeria* sp.	*Apodemus sylvaticus* (wood mouse) - UK
JQ993655	*Eimeria* sp.	*Apodemus agrarius* (striped field mouse) - Czech Republic
JQ993653	*Eimeria vilasi*	*Spermophilus elegans* (Wyoming ground squirrel) - USA
AF080612	*Isospora robini*	*Turdus migratorius* (American robins) - Canada
AF324214	*Eimeria chobotari*	*Dipodomys* sp. (kangaroo rat) - USA
AF307878	*Eimeria arizonensis*	*Peromyscus maniculatus* (deer mice) - USA
AF307880	*Eimeria albigulae*	*Neotoma* sp. (pack rat) - USA
AF307876	*Eimeria antrozoi*	*Antrozous pallidus* (pallid bat) - USA
AF307877	*Eimeria rioarribaensis*	*Myotis ciliolabrum* (western small-footed bat) - USA
AF311642	*Eimeria reedi*	*Perognathus* sp. (pocket mouse) - USA
JQ993645	*Eimeria cahirinensis*	*Acomys dimidiatus* (eastern spiny mouse) - Israel or Jordon
JQ993647	*Eimeria cahirinensis*	*Acomys dimidiatus* (eastern spiny mouse) - Israel or Jordon
JQ993646	*Eimeria cahirinensis*	*Acomys dimidiatus* (eastern spiny mouse) - Israel or Jordon
JQ993654	*Eimeria* sp.	*Acomys* sp. (spiny mouse) - Kenya
AF324213	*Eimeria catronensis*	*Myotis lucifugus* (little brown bat) - USA
AF324215	*Eimeria pilarensis*	*Myotis yumanensis* (mouse-eared bat) - USA
FJ829322	*Eimeria trichosuri*	*Trichosurus cunninghami* (mountain brushtail possum) - Australia
AB544330	*Eimeria gruis*	*Grus monacha* (hooded crane) - Japan
KJ000078	*Eimeria hermani*	*Anser* sp. (domestic goose) - China
AF324217	*Eimeria tropidura*	*Tropidurus delanonis* (Hood Island lizard) - Galápagos Archipelago
GU479655	*Eimeria subepithelialis*	*Cyprinus carpio* (common carp) - Hungary

Table 10.2 COI sequences used in the *Eimeria* spp. phylogenetic analyses

Sequence no.	Species	Host and geographical origin
KT361043	*Eimeria lancasterensis*	Family Sciuridae (squirrel) - Czech Republic
KT361027	*Eimeria sciurorum*	Family Sciuridae (squirrel) - Czech Republic
KT224377	*Isospora manorinae*	*Manorina flavigula* (yellow-throated miner) - Australia
JX839284	*Eimeria tiliquae*	*Tiliqua rugosa* (shingleback skink) - Australia
FJ269361	*Isospora* sp. iSAT5	*Sylvia atricapilla* (Eurasian blackcap) - Sweden
FJ269363	*Isospora hypoleucae*	*Ficedula hypoleuca* (European pied flycatcher) - Germany
JQ993697	*Eimeria nkaka*	*Phataginus tricuspis* (African white-bellied pangolin) - Angola
JQ993686	*Eimeria cahirinensis*	*Acomys dimidiatus* (eastern spiny mouse) - Israel
JQ993706	*Eimeria* sp.	*Apodemus sylvaticus* (wood mouse) - UK
JQ993705	*Eimeria* sp.	*Apodemus flavicollis* (yellow-necked field mouse) - Czech Republic
JQ659301	*Eimeria praecox*	*Gallus* (chicken) - Canada
KR108296	*Eimeria innocua*	Family Phasianidae (turkey, quail, partridge) - Czech Republic
KJ608416	*Eimeria dispersa*	*Meleagris gallopavo* (turkey) - USA
HG793048	*Eimeria dispersa*	*Meleagris gallopavo* (turkey) - Europe
JN192136	*Eimeria trichosuri*	*Trichosurus cunninghami* (mountain brushtail possum) - Australia
HM771680	*Eimeria necatrix*	*Gallus* (chicken) - geographical origin unknown
HM771676	*Eimeria tenella*	*Gallus* (chicken) - geographical origin unknown

10.3 Results

Seventy one quenda were included in this study. This comprised 60 quenda from 12 bushland sites and eleven quenda from urbanised sites (nine quenda from two private residential properties, and two quenda from one private non-residential property).

10.3.1 *Eimeria* spp. identified in Perth quenda

Sporulated oocysts in five faecal samples represented *Eimeria quenda* infection (7.0%, 95% CI 3.1 – 15.5%) (Figure 10.1). Sporulated oocysts in 39 faecal samples represented *Eimeria kanyana* infection (54.9%, 95% CI 43.4 - 66.0%) (Figure 10.2). Oocysts of one novel species of *Eimeria*, formally described in this paper as *Eimeria angustus*, were identified in 32 samples (45.1%, 95% CI 34.0 - 56.6%) (Figures 10.3, 10.4 and 10.5). Sporulated oocysts of a second novel morphotype of *Eimeria* were

identified in two samples (2.8%, 95% CI 0.9 - 9.7%) (Figure 10.6). Mixed *Eimeria* spp. infections were observed in 21 / 71 faecal samples (29.6%, 95% CI 20.2 – 41.1%) - 20 samples had two species of *Eimeria* present and one sample had three species of *Eimeria* present.

Figure 10.1 Photomicrograph of sporulated *Eimeria quenda* oocysts

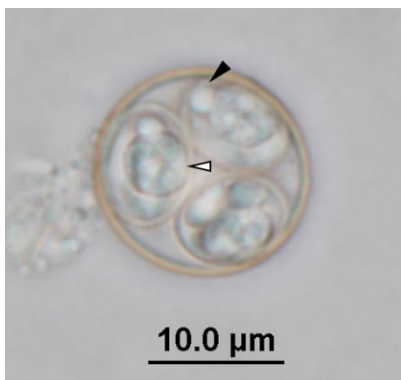**Figure 10.2 Photomicrograph of sporulated *Eimeria kanyana* oocyst**

Figure 10.3 Photomicrograph of sporulated *Eimeria angustus* oocyst
Spherical mass of residuum (◁); large refractile body (◀)
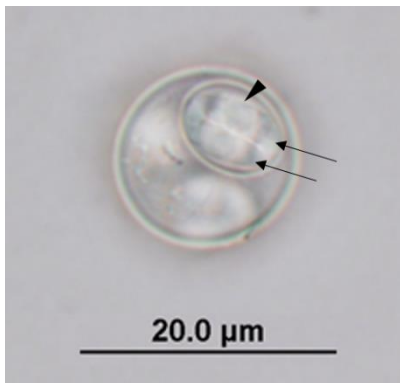
Figure 10.4 Photomicrograph of sporulated *Eimeria angustus* oocyst

Large refractile body (◄); sporozoites (←).

Figure 10.5 Photomicrograph of sporulated *Eimeria angustus* oocyst

Bilaminate cyst wall (⇐).

Figure 10.6 Photomicrograph of a sporulated oocyst of a novel *Eimeria* sp. morphotype

10.3.2   Morphological description of *Eimeria angustus*

Sporulated oocysts are small and spheroidal to subspheroidal. They have a smooth,

bilaminate oocyst wall, without a micropyle. Oocysts contain four oval sporocysts,

which are distinctly compacted within the oocyst. Oocysts do not contain residuum or polar granules. Within each sporocyst, two elongate sporozoites are wrapped around a distinct mass of residuum. Sporocyst surfaces do not have sporopodia or adhering membranes; sporocysts do not have a Stieda body, parastieda body or a substieda body. Each sporozoite contains two refractile bodies; the larger of the two tends to be readily visible, while the smaller refractile body can be difficult to visualise under light microscopy due to the compactness of the cyst obscuring features (Figures 10.3, 10.4 and 10.5). Morphological measurements are described in Table 10.3.

Table 10.3 *Eimeria angustus* morphological measurements

Feature	Mean (µm)	Standard deviation (µm)	Range (µm)
Oocyst wall width	1.02	0.12	0.7 - 1.33
Oocyst length	16.01	1.43	13.07 - 18.52
Oocyst width	15.62	1.31	12.96 - 18.35
Oocyst length: width ratio	1.03	0.02	0.99 - 1.14
Sporocyst length	8.10	0.68	6.20 - 9.74
Sporocyst width	6.03	0.56	4.66 - 7.55
Sporocyst length: width ratio	1.35	0.07	1.11 - 1.52

10.3.3 Genetic characterisation of *Eimeria angustus* and *Eimeria kanyana* at the 18S rRNA locus

A 1216 bp PCR product of *E. kanyana* was successfully amplified and sequenced from the faeces of two quenda - quenda QC14 (isolate KU248088) and QMR13 (KU248089). A 1222 bp PCR product of *E. angustus* was successfully amplified and sequenced from five quenda - quenda QE03 (isolate KU248090), QC04 (KU248091), QC07 (KU248092), QC09 (KU248093) and QC13 (KU248094). Phylogenetic analyses at the 18S rRNA locus using distance, parsimony and maximum likelihood methods produced similar results (Figure 10.7, maximum likelihood tree shown). *Eimeria kanyana* isolates KU248088 and KU248089 shared 98.9% and 99.3% similarity with *E. trichosuri* (FJ829322), respectively. They were grouped in the same clade with *E. trichosuri* (FJ829322), which was identified from mountain brushtail possums (*Trichosurus cunninghami*) in Australia (Power et al., 2009). The five isolates of *E. angustus* (KU248090, KU248091, KU248092, KU248093 and KU248094) grouped in a clade with *E. tropidura* (AF324217), which was identified from *Tropidurus delanonis* (Hood Island lizard) in the Galápagos Archipelago (Aquino-Shuster et al., 1990) and they shared genetic similarities with *E. tropidura* at 96.5%, 96.4%, 96.6%, 96.7% and 96.6%, respectively (Figure 10.7).
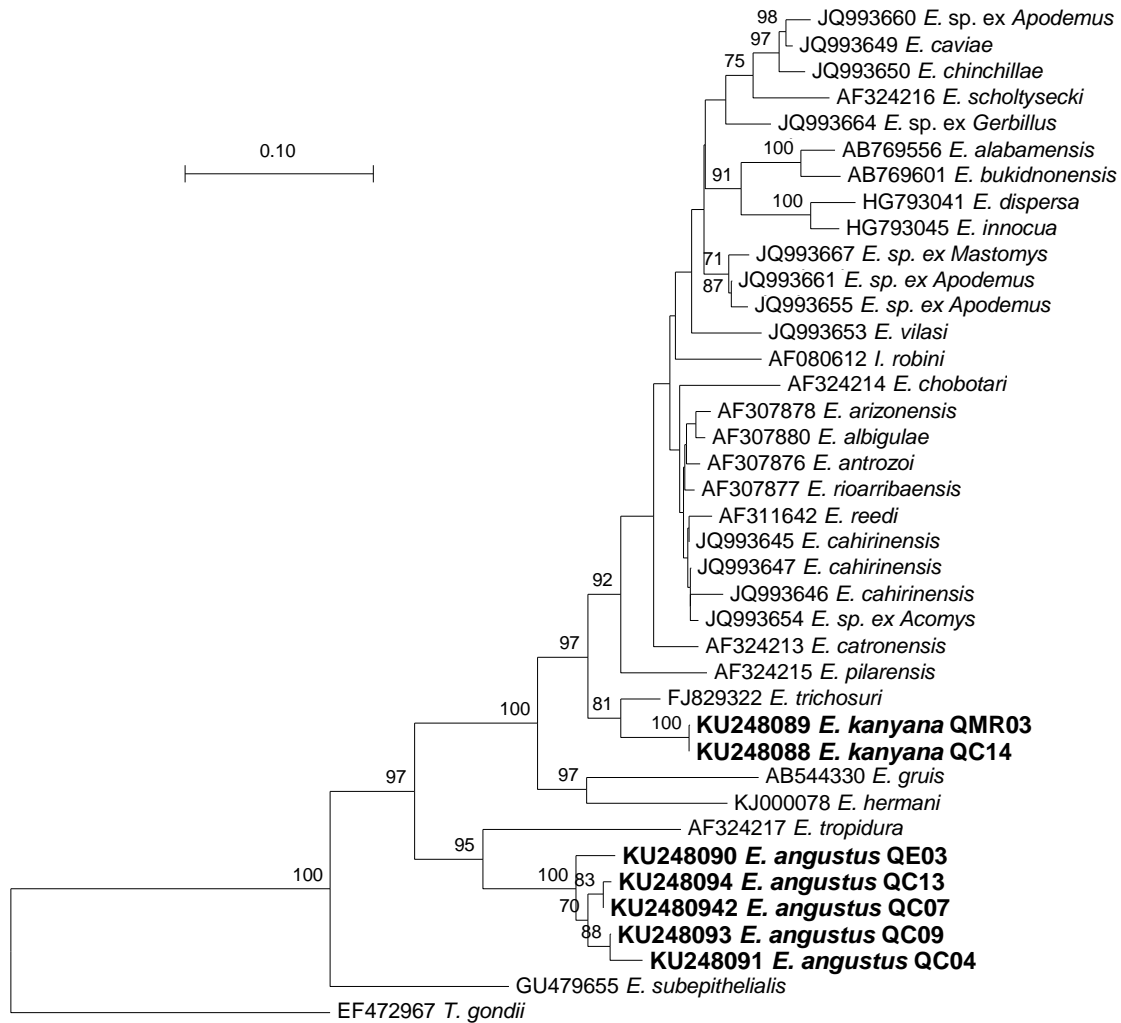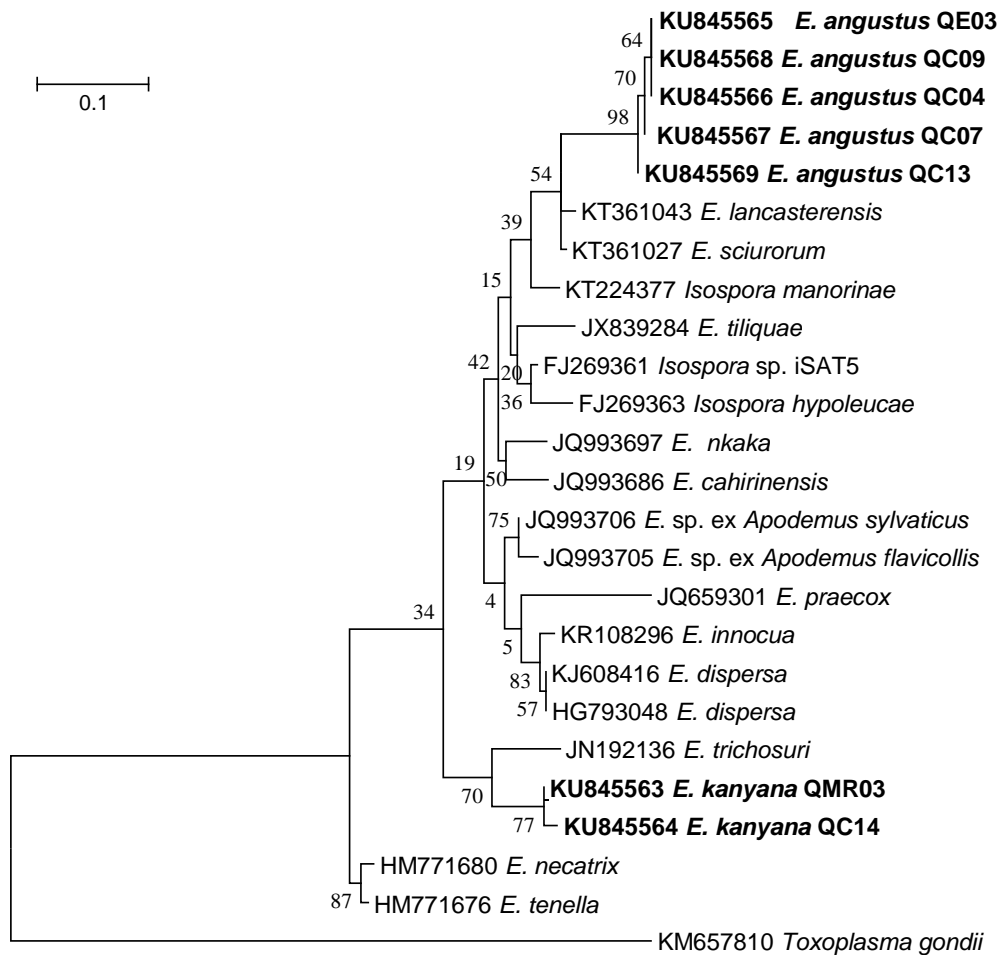
Figure 10.7 Phylogenetic relationships of *Eimeria angustus* and *Eimeria kanyana* isolates obtained in this study, compared to other species of *Eimeria* found in Australia and overseas

Evolutionary history inferred using maximum likelihood analysis of a 1216 bp (*E. kanyana*) or 1222 bp (*E. angustus*) sequence of the 18S rRNA gene. Numbers at nodes indicate % support from 1000 bootstrap replications. *Toxoplasma gondii* is used as the out group.

10.3.4 Genetic characterisation of *Eimeria angustus* and *Eimeria kanyana* at the COI rRNA locus

A 228 bp PCR product of *Eimeria kanyana* was successfully amplified and sequenced from the faeces of two quenda - quenda QMR03 (isolate KU845563) and QC14 (KU845564), while the same size product of *E. angustus* was successfully amplified and sequenced from the faeces of five quenda - quenda QE03 (isolate KU845565), QC04 (KU845566), QC07 (KU845567), QC09 (KU845568) and QC13 (KU845569). Similar to the results from the phylogenetic analysis at the 18S rRNA locus, the two isolates of *E. kanyana* (KU845563 and KU845564) were most closely related to *E. trichosuri* (JN192136) with the similarities at 93.9% and 93.4%, respectively (Figure 10.8). There was no COI sequence from *E. tropidura* in GenBank, and the five *E. angustus* isolates (KU845565, KU845566, KU845567, KU845568 and KU845569) were most closely related to *E. sciurorum* (KT361027), which was originally identified from squirrels from the Czech Republic, with similarities of 95.2%, 95.2%, 95.6%, 95.2% and 96.1%, respectively (Figure 10.8). At the amino acid level, the two COI sequences from *E. kanyana* were identical, as were the five sequences from *E. angustus*, and they differed from each other by only one amino acid (data not shown)*.*

Figure 10.8 Phylogenetic relationships of *Eimeria angustus* and *Eimeria kanyana* isolates obtained in this study, compared to other species of *Eimeria* found in Australia and overseas

Evolutionary history inferred using maximum likelihood analysis of a 228 bp sequence of the COI gene. Numbers at nodes indicate % support from 1000 bootstrap replications. *Toxoplasma gondii* is used as the out group.

10.4   Discussion

This study suggests that *Eimeria* spp. infections are highly prevalent in Perth quenda.

Indeed, prevalences may have been underestimated, as not all coccidian oocysts

consistent with *Eimeria* spp. sporulated in all samples. Although we cannot exclude

bias due to the use of non proportionate sampling methodology, which was utilised out

of practical necessity, these findings concur with the prevalence of unsporulated

coccidian oocysts (consistent with *Eimeria* spp.) observed in a survey of quenda

sampled post mortem, suggesting that the use of traps is unlikely to have been a source of selection bias (Hillman et al., unpublished results). Consistent with these findings, moderate to high *Eimeria* spp. prevalences have been recorded in other free-ranging Australian marsupial populations (Mykytowycz, 1964; Barker et al., 1988a,c; Barker et al., 1989; O'Callaghan et al., 1998; Bennett et al., 2006; Yang et al., 2012, Austen et al., 2014).

Four putative species of *Eimeria* were found in quenda in this study, with only two of these species having been previously described. To our knowledge, this is the first time that *E. kanyana* has been documented in quenda, expanding the known host range of this parasite to two genera of peramelids. This is an unusual finding, given the typical host specificity of *Eimeria* spp. (Joyner, 1982). *Eimeria* spp. have been previously documented parasitising multiple host species within the same marsupial genus (Barker et al., 1988a; Barker et al., 1989; O'Callaghan and O'Donoghue, 2001; Power et al., 2009), but not multiple marsupial hosts spanning different genera. The sporulation of two undescribed species of *Eimeria* is not unexpected, based on findings of Bennett and Hobbs (2011), who observed a novel morphotype of *Eimeria* spp. when describing *E. quenda*, and reported unpublished archives of two morphologically distinct *Eimeria* spp. oocyst types isolated from quenda hosts. We have described the most prevalent undescribed species in our study as *E. angustus*.

Our data suggest that concurrent infection with multiple species of *Eimeria* is relatively common in quenda. Furthermore, we may have underestimated the prevalence of mixed infections, as in some samples there were unsporulated oocysts of mixed morphology, but oocysts of one or both morphotypes failed to sporulate. The relatively common occurrence of mixed infections concurs with findings from studies of *Eimeria* spp. infections in the Australian marsupial hosts Pearson Island rock wallabies (*Petrogale lateralis pearsoni*) (O'Callaghan et al., 1998) and quokka (*Setonix brachyurus*) (Austen et al., 2014). Additionally, though mixed infections were not

specifically discussed, the findings of Mykytowycz (1964) and Barker et al. (1988a) indicated the presence of mixed *Eimeria* spp. infections in various species of macropods. In contrast, a study of Bolivian marsupials rarely identified mixed *Eimeria* spp. infections (Heckscher et al., 1999). The implications of mixed *Eimeria* spp. infections on the pathogenicity in quenda is uncertain. Studies in other host species have variably identified synergistic, antagonistic or no effects on pathogenicity from mixed infections of *Eimeria* spp. (e.g. Hein, 1976; Cornelissen et al., 2009; Moreno et al., 2013). It has been suggested that this may depend on whether or not the sites of infection for the various *Eimeria* species in the small intestine overlap (Catchpole and Norton, 1979), which is not known regarding *Eimeria* spp. in quenda.

This study did not identify any parasites of the Family Eimeriidae that are of known anthropozoonotic significance in Perth quenda.

10.4.1 *Eimeria angustus* taxonomic summary

Host type: *Isoodon obesulus* - quenda (syn. southern brown bandicoot). *Eimeria angustus* was obtained from both subadult and adult quenda.

Type locality: *Eimeria angustus* was sporulated from quenda faecal samples obtained from eleven sites throughout the greater Perth region, Western Australia.

Sporulation time: unknown

Site of Infection: unknown

Prepatent and patent periods: unknown

Material deposited: formalin preserved and ethanol preserved sporulated oocysts, and oocyst photosyntypes, have been deposited at the Western Australian Museum (specimen registration no. WAM Z68786)

Etymology: The specific epithet *angustus* is from the Latin, meaning "contracted, small, not spacious" – a distinctive feature of this species of *Eimeria.*

10.4.2 *Eimeria angustus* morphology, as compared to other known *Eimeria* spp. of bandicoots (Family Peramelidae)

Eimeria angustus is substantially smaller than *E. quenda,* and has a distinct spherical mass of residuum within the sporocysts, unlike *E. quenda*.

Eimeria angustus tends to be smaller than *E. kanyana*, though the size ranges of these species do overlap. Unlike *E. kanyana, E. angustus* lacks Stieda bodies and polar granules, and has a distinct spherical mass of residuum within the sporocysts.

There is another novel morphotype of *Eimeria* in quenda, observed previously (A. Elliot, Murdoch University - pers. comm.) and as part of the current study. In specimens sporulated as part of this study, this novel *Eimeria* sp. morphotype was observed to be substantially larger and distinctly ovoid, compared to *E. angustus*. Stieda bodies are present in this novel morphotype, which are not found in *E. angustus*.

10.4.3 Genetic characterisation of *Eimeria kanyana and Eimeria angustus*

Earlier phylogenetic analyses of different marsupial *Eimeria* spp. at the 18S rRNA locus indicate that they form a monophyletic group, separate from species found in eutherians and birds (Power et al., 2009; Yang et al., 2012). These studies also suggested that more detailed phylogenetic inferences, within the clade found in marsupial hosts, would be assisted by analyses at multiple loci, hence the use of both the 18S rRNA and COI loci in this study.

Across both loci tested, *E. kanyana* was identified as a clade most closely related to *E. trichosuri,* parasitic in mountain brushtail possums (*Trichosurus cunninghami),* another marsupial species endemic to Australia (Power et al., 2009). The phylogenetic placement of *Eimeria angustus* was not so clear. As there are limited COI sequences in GenBank®, and no other *Eimeria* spp. parasitic in hosts of the Order Peramelemorphia

have been genetically characterised prior to this study, it is not possible at this stage to confirm the phylogenetic placement of *E. angustus*. However, it seems that *E. angustus* is not closely related to *E. kanyana,* also parasitic in peramelid hosts. This is unexpected, given previous studies have suggested monophyletic origin for *Eimeria* spp. parasitising marsupials (Power et al., 2009; Yang et al., 2012). The putative relationship with *E. tropidura* seems especially unusual, as this species was isolated from a reptile host. As lizards and other reptiles are not known to be a typical component of *I. obesulus* diets (Heinsohn, 1966; Quin, 1998), and *E. angustus* had a relatively high prevalence in quenda, it seems unlikely that *E. angustus* was being shed in quenda faeces after consumption of a reptilian host. Additionally, the large number of oocysts observed in many samples, and the fact that all were passed unsporulated, suggests that quenda were amplifying the infection rather than acting as a vector.

10.5   Acknowledgements

10.6 References

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Chapter 11

General discussion

11. General discussion

11.1   Introduction

The research comprising this thesis aimed to investigate the impacts of urbanisation on parasitic infections (particularly anthropozoonoses, with a focus on *Giardia* spp. and *Toxoplasma gondii*), morphometrics and reproduction in quenda and brushtail possums in the greater Perth region, Western Australia. In doing do, it has provided the first observations of the impact of urbanisation on marsupials in Perth. The sampling strategy and, regarding quenda, the number of animals sampled, are more comprehensive than any previous studies investigating the impact of urbanisation on marsupials.

Findings suggest that quenda in urbanised environments are more likely to be overweight or obese than those inhabiting bushland, and that adult female quenda may have reduced fecundity in urbanised environments compared to bushland. From a parasitological perspective, quenda in the greater Perth region tended to be parasitised by a range of gastrointestinal and ecto- parasites in both urbanised environments and bushland, with evidence of an increased odds of infection with certain parasites in quenda trapped in urbanised environments compared to bushland. Variations in ectoparasite intensities of infection and parasite communities in quenda trapped between these environments were also documented. In contrast, brushtail possums in the greater Perth region tended to have low gastrointestinal and ecto- parasite prevalences and intensities of infection, with a lower prevalence of tick infections in brushtail possums trapped in urbanised environments compared to bushland.

This research has also formally described two parasite species (*Giardia peramelis* and *Eimeria angustus*), identified nine parasite taxa infecting quenda for the first time (*Giardia canis* genotype D, *Eimeria kanyana*, *Echidnophaga myrmecobii*, *E. gallinacea*, *Lucilia* sp., *Calliphora* sp., *Ixodes australiensis*, *Amblyomma* spp. and *Haemaphysalis humerosa*), identified two parasite taxa infecting brushtail possums for the first time (*Pygiopsylla tunneyi* and *Liponyssoides* sp.), and expanded the known geographic distribution of a range of additional parasite genera or species of quenda and / or brushtail possums to include the greater Perth region. This research also provides the first validation studies of parasite detection tests for use in detecting *Toxoplasma gondii* infection in quenda and brushtail possums, and *Giardia* spp. infection and gastrointestinal helminth infections in quenda. Further, the research has documented a range of interactions that occur between humans and urban dwelling marsupials in Perth, which are of public health significance, and also presents the first epidemiological data of *T. gondii* seroprevalence and cat management factors relevant to public health in Perth pet cats.

11.2 Summary of observations from and hypotheses generated by this research

11.2.1 The impacts of urbanisation on parasitism, morphometrics and reproductive success are likely to vary in nature and magnitude across different wildlife species, different geographic (ecologic) locations, and different parasite taxa

In comparing our findings between quenda and brushtail possums in Perth, and to those of other research on mammals, it is clear that the impacts of urbanisation on one species in one geographic (ecologic) location cannot be used to make assumptions about the impact of urbanisation on other species or in other geographic (ecologic) locations.

Regarding the impacts of urbanisation on parasite epidemiology, morphometrics and reproductive success, it is probable that the impacts differ between wildlife species in the same or comparable locations, based on factors such as differences in physiology, diet, whether the species is arboreal or terrestrial, whether the species is of a social or solitary nature, and whether the species is relatively gregarious or aloof in interacting with cohabiting domestic animals and humans in urbanised environments.

Similarly, impacts of urbanisation on the same species may vary between geographic locations due to differing local environmental conditions, which may influence factors such as comparative resources available in non-urbanised and urbanised environments, the presence of sympatric wildlife species relevant to infection epidemiology, wildlife population densities and survival of infectious agents in the local environmental conditions.

Additionally, differences in the biology of various parasite species, and differences in parasite species infecting a host species across different geographic (ecologic) locations, may modify the impacts of urbanisation on parasitism between host species, and between the same host species in differing locations.

An example of apparent discrepancies in the impact of urbanisation on parasitism is that whilst prevalence of ticks on brushtail possums in urbanised environments in Perth was lower than in bushland (Chapter 6), ticks were more prevalent on quenda in urbanised environments in the same region, with quenda in urbanised environments having increased odds of infection with *Ixodes fecialis* (Chapter 7). Brushtail possums may spend more time on the ground foraging in bushland, where domestic animals are less of a threat, and in doing so they may be more likely to be exposed to ticks. In contrast, quenda are terrestrial in both environments. Further, these marsupials were parasitised by different species of ticks, and differences in the biology of these ticks may modify the impacts of urbanisation on the epidemiology of tick parasitism between these hosts. Considering particular genera or species of ticks involved, in Perth *Amblyomma* spp. infection was more prevalent in brushtail possums in bushland, whereas there was no difference in the prevalence of *Amblyomma* spp. infection between quenda trapped in urbanised environments compared to bushland, possibly reflecting modified behaviour of possums in urbanised environments, such as spending less time foraging on the ground.

Similarly, of two closely related tick species parasitic on Perth quenda, *I. fecialis* was markedly more prevalent in urbanised areas than bushland, supported by substantially increased odds of infection in urbanised environments. In contrast, the prevalence of *I. australiensis* did not vary between these environments. This may be driven by differences in the biology of the two tick species that moderate the impacts of urbanisation, but the lack of knowledge of the biology of native Australian ticks that are not considered important to human and domestic animal health precludes further speculation.

Further, ectoparasite findings of brushtail possums trapped in urbanised environments in Perth (Chapter 6) differ to those of brushtail possums trapped in an urbanised environment in Sydney, Australia, where tick infections were more prevalent than in the Blue Mountains bushland brushtail possum population to which they were compared (Webster et al., 2014). Varying environmental conditions both between urbanised Perth and urbanised Sydney, and between the urbanised Sydney site and the Blue Mountains bushland site to which it was compared, may explain these differing observations.

Considering anthropozoonotic parasite infections, such infections were more prevalent in Perth quenda than in brushtail possums (Chapters 6 and 7). Quenda are often active during the day or early evening in Perth, and can be relatively gregarious in interacting with humans and domestic animals (Chapter 3; Howard et al., 2014), compared to the nocturnal and generally aloof brushtail possums. Their relative boldness may put them at increased risk of exposure to human and domestic pet infections, compared to less gregarious species such as brushtail possums.

Likewise, this research identified quenda trapped in urbanised environments to be more likely to be overweight or obese that those in bushland (Chapter 7), while the data from the brushtail possums sampled within the same geographic region, although limited by the small sample size, did not provide any evidence that this may similarly be the case for them (Chapter 6). This may be attributable to a number of factors, including that quenda are relatively gregarious in interactions with humans and domestic animals (Chapter 3; Howard et al., 2014), compared to the nocturnal and generally aloof brushtail possums, and thus may be more likely to be offered food by members of the public or successfully scavenge food. Additionally, the terrestrial habits of quenda, compared to brushtail possums' primarily arboreal habits, may make them more likely to have access to high-energy anthropogenic food stuffs such as pet food. Long-nosed bandicoots in suburban backyards in the greater Sydney region have similarly been identified as having increased body condition, compared to those in bushland (Dowle, 2012). Other mammalian wildlife research worldwide has documented relatively increased body weight associated with urbanisation, for example grey foxes (*Urocyon cinereoagenteus*) in New Mexico, USA (Harrison, 1997), San Joaquin kit foxes (*Vulpes macrotis mutica*) in California, USA (Cypher and Frost,1999), Key deer (*Odocoileus virginianus clavium*) in Florida Keys, USA (Harveson et al., 2007) and wild boar (*Sus scrofa*) in Barcelona, Spain (Cahill et al., 2012). However, these studies did not also compare skeletal morphometrics or body condition, to enable assessment of whether this relatively increased body weight was likely due to increased body condition in urbanised environments.

Finally, while these research findings suggest that urbanisation may negatively impact the fecundity of adult female quenda (Chapter 7), available evidence suggests the opposite is true for brushtail possums in urbanised Launceston, Tasmania (Statham and Statham, 1997). This discrepancy may be attributable to differing species population densities proportionate to available resources in urbanised environments *vs.* bushland between these two locations, and possibly differences in reproductive physiology. It also must be considered that in comparing quenda trapped in bushland and urbanised environments within the greater Perth region, rather than comparing Perth quenda to quenda in geographically remote bushland that may vary in indigenous ecosystem structure and therefore available resources for quenda than bushland in the Perth area, this research has avoided such potential bias in comparing fecundity of adult female quenda trapped in urbanised environments *vs.* bushland. This bias cannot be excluded where observations have been made comparing marsupials in urbanised environments to bushland marsupials in different geographical locations with varying indigenous ecosystem structure. The effects of urbanisation on the reproduction of other mammalian wildlife studies worldwide has been demonstrated to vary. For example, seasonal decreases in fecundity associated with urbanisation have been documented in Key deer (Harveson et al., 2007), whilst San Clemente island foxes (*Urocyon littoralis clementae*) that spent a greater proportion of time in urban areas have a greater probability of reproducing (Gould and Andelt, 2001).

11.2.2 Anthropozoonotic gastrointestinal parasites and macroscopic ectoparasites are not prevalent on quenda and brushtail possums in the greater Perth region

Though parasitism is common in Perth quenda, and also occurs in Perth brushtail possums, the parasites identified, both in terms of prevalence and intensity of infection, were dominated by parasites that were either known to be, or deemed likely to be (in consideration of all study findings), native species that are not known to commonly infect humans or pets.

This reflects other research findings regarding parasitism in urban dwelling wildlife in Australia (Hill et al., 2008a; Thompson et al., 2010; Webster et al., 2014), with the exception of the paralysis tick *Ixodes holocyclus* in brushtail possums, southern brown bandicoots and long-nosed bandicoots in Sydney (Dowle, 2012; Webster et al., 2014), and the stick-fast flea *Echidnophaga myrmecobii* in brushtail possums in Sydney (Webster et al., 2014).

11.2.3   Do native species of parasite outcompete species that did not evolve in Australia with the native hosts?

The *Giardia* spp. findings in quenda (Chapter 8) suggest that native parasites, adapted to infect native animals, may generally outcompete related introduced species from humans and domestic animals. Evidently, *Giardia* spp. transmits readily in quenda populations in Perth, as the prevalence of the host-adapted *Giardia peramelis* was relatively high and it was isolated from quenda at many locations (Chapter 8). Quenda are also known to be susceptible to infection with *G. canis,* host-adapted to canines, and *G. duodenalis*, which is capable of infecting humans and a wide range of other animals (Thompson et al., 2010). Infection with such *Giardia* species are prevalent in Australian pet dogs (Palmer et al., 2008), and given the likelihood that quenda in urbanised environments are exposed relatively regularly to such faecal borne parasites originating from domestic pet faeces, it seems remarkable that these different species of *Giardia* are not more prevalent in quenda.

Similarly, this research found ectoparasitic infections in Perth brushtail possums (Chapter 6) and Perth quenda (Chapter 7) were dominated by native species of ectoparasite, despite the findings that they are susceptible to certain domestic animal ectoparasites (Chapters 6 and 7). Similarly, Dowle (2012) and Webster et al. (2014) found that ectoparasitism in southern brown bandicoots and long-nosed bandicoots, and brushtail possums (respectively) in urbanised environments in Sydney was dominated by native ectoparasite species. This is despite the fact that both quenda and brushtail possums are known to be susceptible to infection with a variety of domestic animal flea species (Dunnet and Mardon, 1974), including *Ctenocephalides felis*, which dominates flea parasitism in Australian pet dogs and cats (Šlapeta et al., 2011).

Furthermore, Hill et al. (2008a) found that while *Cryptosporidium* spp. infections were prevalent in brushtail possums in Sydney, all were of novel genotypes that are not known to infect humans or pets. However, it is not known whether brushtail possums are susceptible to human and domestic animal *Cryptosporidium* spp. infections.

11.3   Limitations associated with this research

The primary limitations of this research are: the use of non-proportionate sampling; the lack of temporal sequence; small sample sizes, particularly regarding brushtail possums; potential for misclassification regarding categorising animals as 'bushland' animals; and the inability to measure and investigate the role of population density in the various parasitic and reproductive discrepancies identified between marsupials trapped in urbanised environments and bushland.

11.3.1 Use of non-proportionate sampling

Non-proportionate sampling had to be employed, as proportionate sampling strategies were not feasible for a number of reasons. The primary issue in trapping was that population distributions of quenda and brushtail possums across both urbanised environments and bushland in the greater Perth region are heterogeneous and not well documented. Additionally, trapping on private properties needed to be undertaken with property owners' consent. Finally, undertaking trapping at a site does not guarantee successfully trapping local animals, or trapping a sample of animals that necessarily represents the local population.

However, faecal samples collected post mortem from 50 quenda and (quenda obtained opportunistically from the greater Perth region - not via trapping) were examined by the same methods as trapped quenda (Chapter 4), and the prevalences of all parasites were similar to the overall findings of quenda trapped in the greater Perth region (A. Hillman – unpublished data). This suggests, though is not conclusive, that the use of traps was not a source of sampling bias in these results.

Non-proportionate sampling strategies are commonly employed in studies of wildlife parasitism worldwide (e.g. Lehrer et al., 2010; Watts and Alexander, 2012; Webster et al., 2014).

11.3.2 Lack of temporal sequence

Temporal sequence would be valuable for estimating the incidence of infections. However, lack of temporality is considered less of a concern in the comparisons made between animals trapped in urbanised environments and bushland, as reverse causality is considered unlikely. For example, it is considered unlikely that becoming obese would cause a quenda to disperse to an urbanised environment, or that a certain parasitic infection would cause an animal to alter its home range from bushland to urbanised environment (or vice versa).

Research of wildlife parasitism commonly lacks temporal sequence, or if repeat observations are obtained they are too uncommon to enable meaningful statistical analyses involving temporal sequence (e.g. Hill et al., 2008a,b; Turner and Getz, 2010; Gaetano et al., 2014).

To expand on the findings of this thesis, future research must include observations with temporal sequence. Given the baseline data provided by this research, sample size calculations should be facilitated and allow for efficient planning of such projects through relatively accurate prediction of resource requirements.

11.3.3 Small sample size

The small sample sizes, particularly regarding brushtail possums, limited statistical extrapolations by reducing statistical power, and reducing precision in prevalence estimates and measures of effect. Small samples are a common limitation of wildlife studies, and this quenda sample size is substantially larger than all previous research undertaken to investigate the impacts of urbanisation on urban dwelling marsupials (e.g. Hill et al., 2008a,b; Dowle, 2012; Webster et al., 2014) and many other cross sectional studies investigating the impacts of urbanisation on mammalian wildlife worldwide (e.g. Harrison, 1997; Cypher and Frost, 1999; Johnston et al., 2010; Lehrer et al., 2010).

Our brushtail possum sample size was particularly limited by the difficulty in trapping brushtail possums in urbanised environments using cage traps set on the ground. Alternative modes of capture may be more suitable for this species in future research in urbanised environments.

11.3.4 Potential for misclassification regarding categorising animals in respect of exposure to urbanisation

In comparing animals trapped in urbanised environments and bushland, we were not able to ensure that the home range of all bushland animals did not encompass urbanised sites. However, the sampling strategy was specifically designed in an attempt to minimise the potential for such misclassification on findings, and any misclassification present would have biased our findings to the null. This would have resulted in a relatively increased risk of Type II error, entailing a conservative interpretation of data. Thus, statistical findings against the null hypothesis are considered valid, though possibly underestimated; however, Type II error needs to be considered where there was no evidence against the statistical null hypothesis.

Importantly, by trapping bushland in the same geographic region as the urbanised sites, this research avoided a number of potentially biasing factors that may have occurred in comparing animals trapped in urbanised environments in the greater Perth region to geographically (and ecologically) remote bushland completely isolated from urbanisation. This is a substantial limitation of other research undertaken to investigate the impacts of urbanisation on other marsupial species (e.g. Hill et al., 2008a,b; Webster et al., 2014).

Additionally, in trapping a relatively large number of bushland sites throughout the greater Perth region (and likely increasing the risk of bushland misclassification in doing so) we have avoided disparate bushland parasitism findings, such as those observed regarding *Haemaphysalis humerosa* infection at one bushland site in this study (Chapter 7) and tick parasitism between two bushland sites in Sydney, Australia, in Dowle (2012), from being presumed representative of all bushland in the study area. The same cannot be said for studies where few bushland locations were sampled in making comparisons (e.g. Hill et al., 2008a,b; Webster et al., 2014 - one bushland location each).

11.3.5 The inability to measure population density

The inability to measure population density, and consider density in the statistical analyses, precluded objective assessment of population density as an explanatory factor regarding various study findings associated with urbanisation. Safety of the trapped animals had to be a priority in trapping both urbanised sites and bushland that is accessible to humans, and this did not allow for trapping layouts optimal for such measurements.

11.4 Concluding comments

To have wildlife remaining in urbanised environments is a privilege, with benefits including raising awareness of and fostering an interest in wildlife amongst humans residing in urban areas, and the pleasure which many people derive from observing wildlife on their doorstep. Public health risk in this scenario is to be understood, not hyped up based on speculation. Importantly, the adverse consequences on wildlife are not to be dismissed – they deserve attention in public education, and in the management of pets and of human interaction with wildlife. This is important for the health of wildlife, humans and domestic animals cohabiting in urbanised environments.

11.5 References

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Appendix A   Parasites of quenda (*Isoodon obesulus*) in Perth, Western Australia

A.1   Gastrointestinal parasites of Perth quenda

A.1.1   *Giardia peramelis* and *Cryptosporidium* sp. observed on immunofluorescence microscopy

Giardia peramelis
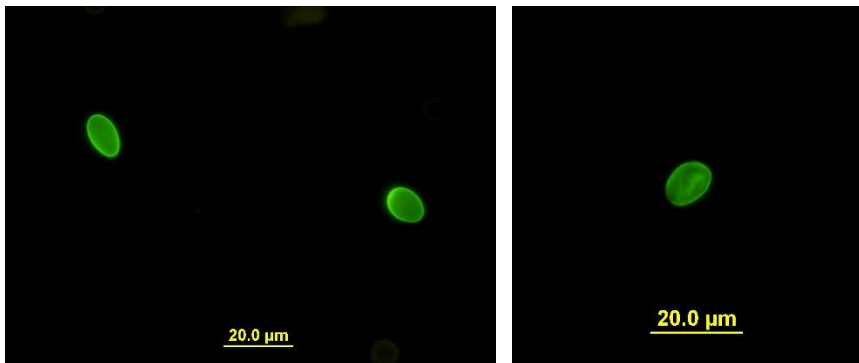
Cryptosporidium sp.
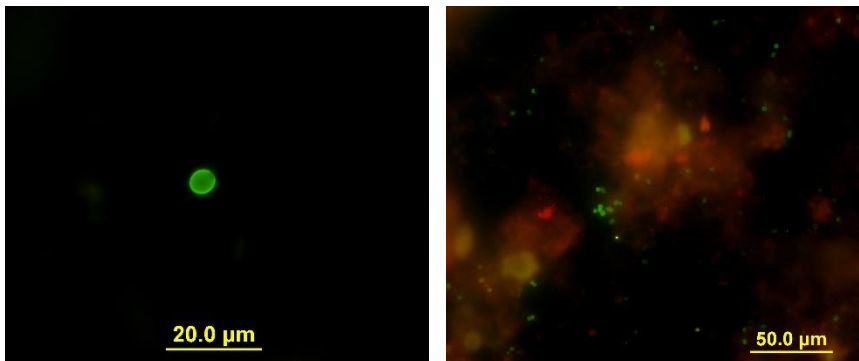
Cryptosporidium sp. (left) and *Giardia peramelis* (right)
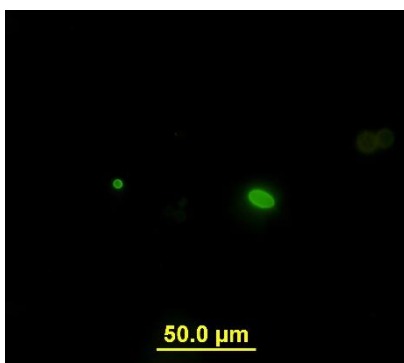
A.1.2 Gastrointestinal protozoans observed on light microscopy, by centrifugal faecal flotation

Giardia peramelis
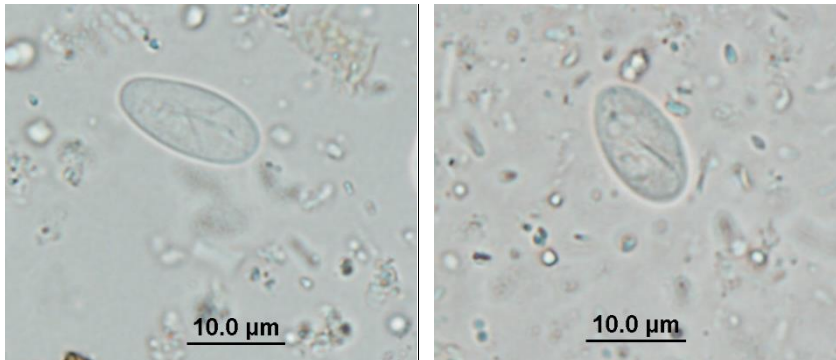
Entamoeba spp.
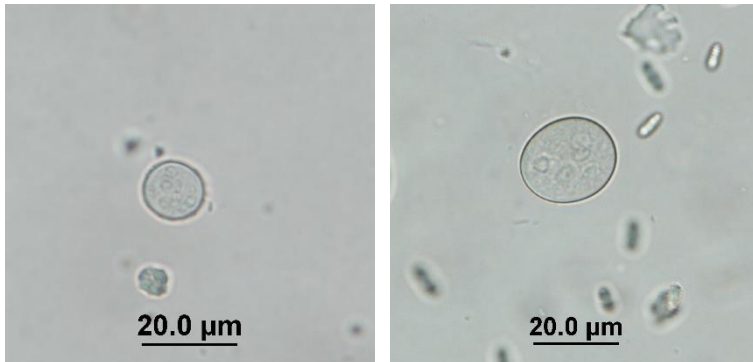
Eimeria kanyana:

Unsporulated oocyst   Sporulated oocyst

Eimeria angustus:

Unsporulated oocyst   Sporulated oocyst
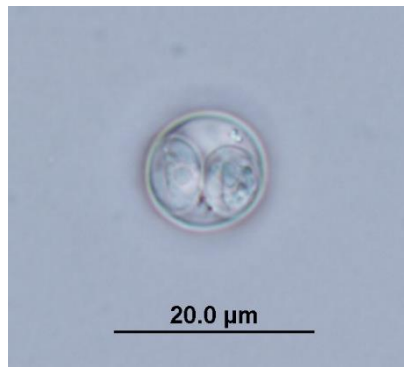
Eimeria quenda:

Unsporulated oocysts   Sporulated oocysts

Novel *Eimeria* sp.:

Sporulated oocyst
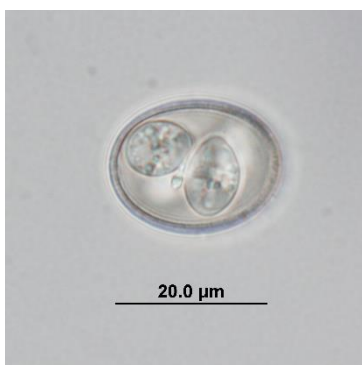
A.1.3　　　Gastrointestinal helminth eggs observed on light microscopy, by centrifugal faecal flotation

Strongyle eggs (Infraorder Rhabditomorphia):

Unembryonated egg　　　　　Embryonated egg

Approximate dimensions (range):　length: 52.5 µm – 72.5 µm
width:　30 µm – 45 µm

Labiobulura spp. eggs:

Approximate dimensions (range):　length:　52.5 µm – 65 µm
width:　45 µm – 60 µm

Linstowinema spp. eggs:

Approximate dimensions (range):　length: 35 µm – 45 µm
width: 32.5 µm – 45 µm

Physaloptera spp. eggs:
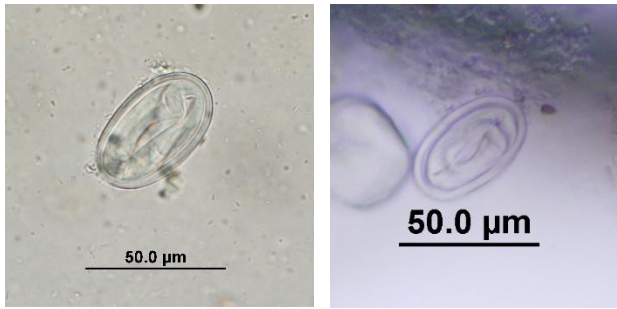
Approximate dimensions (range): length: 45 µm – 55 µm
width: 27 µm – 32.5 µm

Strongyloid eggs (Family Strongyloididae):
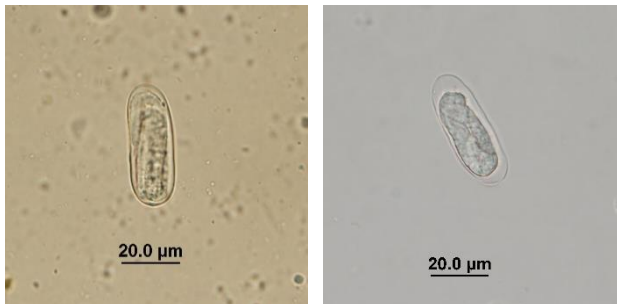
Approximate dimensions (range): length: 40 µm – 45 µm
width: 15 µm – 20 µm

Trichuris spp. eggs:

Approximate dimensions (range): length: 47.5 µm – 60 µm
width: 22.5 µm – 27.5 µm

Potorolepis spp. eggs:
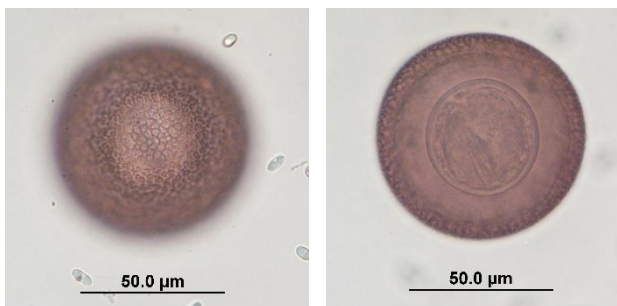
Approximate dimensions (range): length: 65 µm – 102.5 µm
width: 65 µm – 80 µm

A.2 Ectoparasites of Perth quenda

A.2.1 Fleas

Acedestia chera:

Pygiopsylla tunneyi:

Stephanocircus dasyuri:

Echidnophaga myrmecobii
(photographs of a specimen obtained from a brushtail possum)
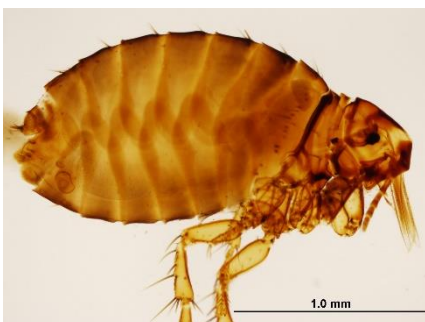
Echidnophaga gallinacea

(photographs of a teaching specimen)

A.2.2　　　Lice

Boopia bettongia

A.2.3   Ticks

Ixodes fecialis female
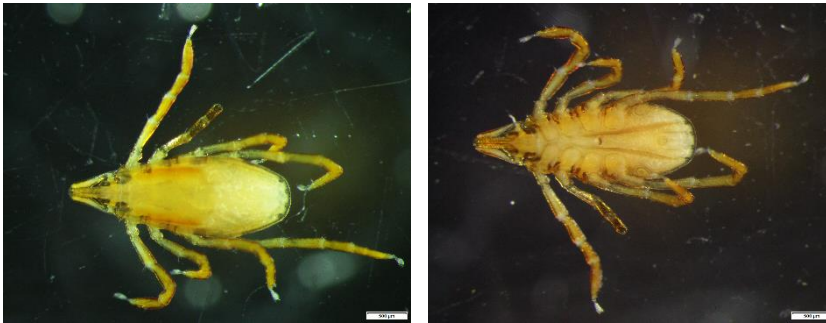
Ixodes fecialis male

Ixodes australiensis female
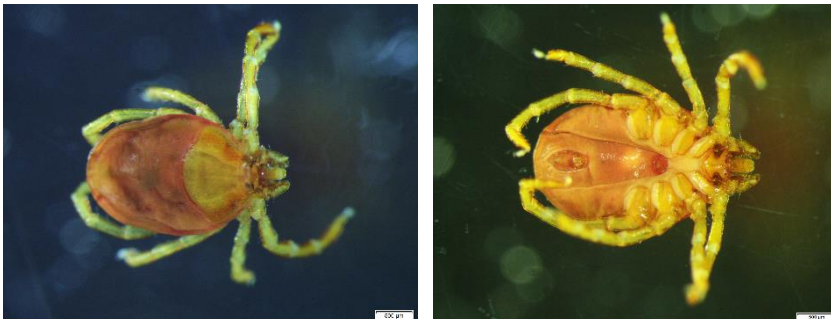
Amblyomma sp. nymph
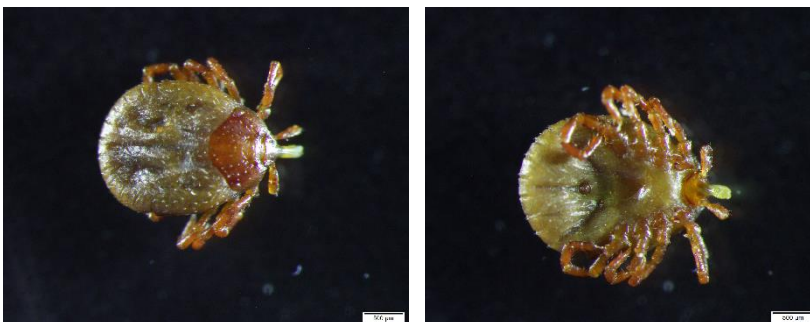
Haemaphysalis humerosa female

Haemaphysalis humerosa male

A.2.4 Mites

Haemolaelaps marsupialis

Gymnolaelaps annectans
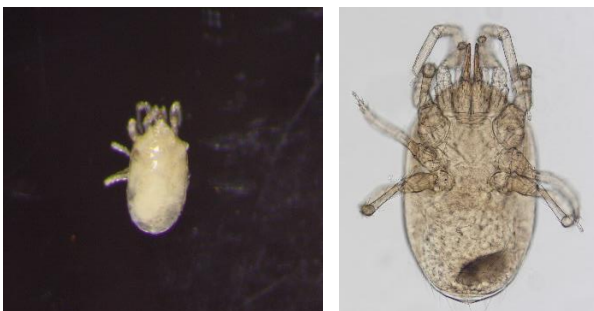
Mesolaelaps australiensis
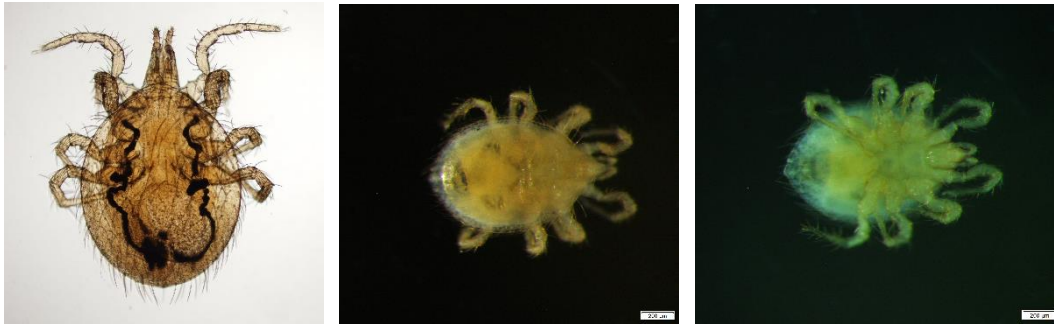
Mesolaelaps antipodianus


Trombiculid mites (Suborder Prostigmata) - in situ*:*

Peri-scrotal region: Peri-pouch:

Appendix B   Parasites of brushtail possums (*Trichosurus vulpecula*) in Perth, Western Australia

B.1   Gastrointestinal parasites of Perth brushtail possums

Dimensions observed at 400x magnification.

B.1.1   Gastrointestinal protozoans observed on light microscopy, by centrifugal faecal flotation

Entamoeba sp. cysts
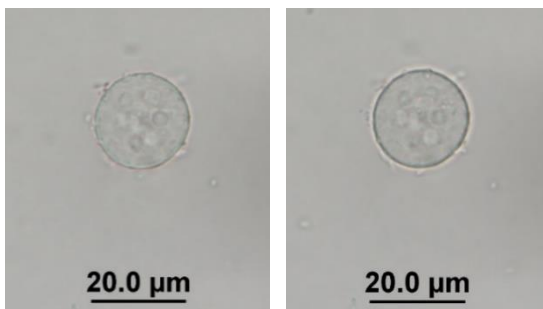
Length 12.5 - 20 µm; width 12.5 - 20 µm

Unsporulated coccidian oocyst (Family Eimeriidae) (morphologically consistent with *Eimeria trichosuri*)

Length 32.5 - 45 µm; width 17.5 - 25 µm

Unsporulated coccidian oocyst (Family Eimeriidae)

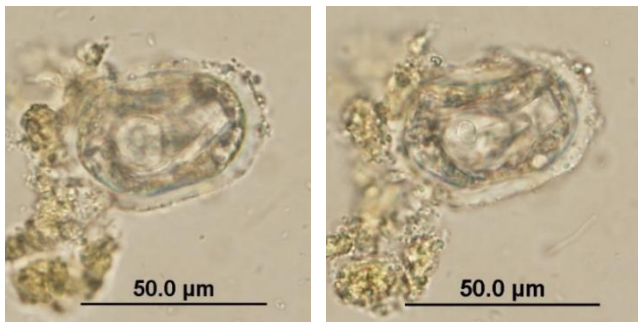Length 17.5 - 25 µm; width 15 - 22.5 µm

B.1.2 Gastrointestinal helminth eggs observed on light microscopy, by centrifugal faecal flotation

Strongyle eggs (Infraorder Rhabditomorphia)

Length 52.5 – 100 µm; width 30 – 45 µm

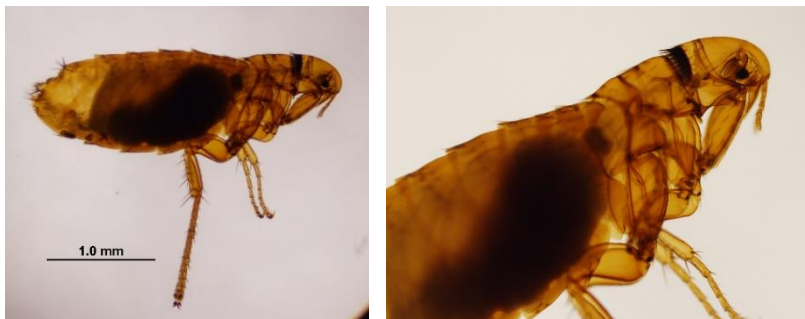Unidentified acanthocephalan egg (Phylum Acanthocephala)

Length 55 µm ; width 37.5 µm

B.2   Ectoparasites of Perth brushtail possums

B.2.1   Fleas

Choristopsylla ochi

Echidnophaga myrmecobii

Pygiopsylla tunneyi

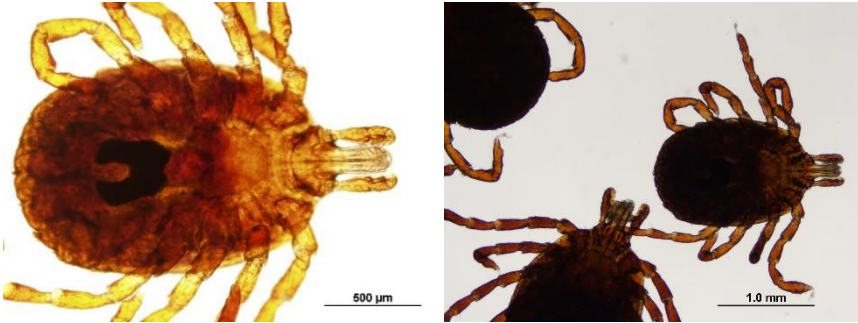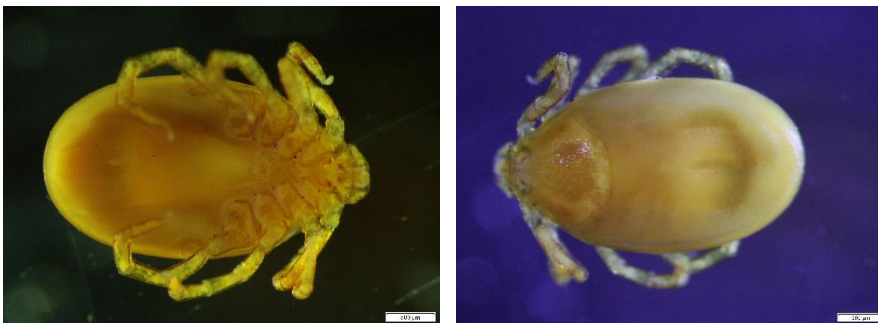## B.2.2 Ticks

Amblyomma spp. nymphs

Haemaphysalis bremneri

Ixodes tasmanii

B.2.3 Mites

Liponyssoides sp.