

An investigation into the role *Toxoplasma gondii* may
play in the health of the southern brown
bandicoot (*Isoodon obesulus obesulus*) and an
assessment of environmental contamination with *T.*
gondii.



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Photograph on Title Page: Sculpture of a southern brown bandicoot placed in the Royal Botanic Gardens Cranbourne to raise awareness about the resident population of the nationally endangered southern brown bandicoots.

Artists: Darrell Cordell, Vanessa Ellis, Trina Gaskell and Cliff Dolliver

Sculpture formed from *Kunzea ericoides*

Abstract

The southern brown bandicoot (*Isoodon obesulus obesulus*), a small, ground-dwelling marsupial, is listed as 'endangered' under the Australian Environment Protection and Biodiversity Conservation Act 1999. While many factors contributing to population decline are understood, in particular predation and loss of high-quality, connected habitat, there is a lack of knowledge about other threatening processes, including disease, which has contributed to declines in other small mammal species.

Toxoplasma gondii, a protozoan intracellular parasite, excreted into the environment by cats, has been shown to cause clinical disease, including death, in many small and medium sized captive and free-ranging marsupials, including the eastern barred bandicoot (*Perameles gunnii*). Little is known of its effect on southern brown bandicoot populations. This study aimed to investigate the significance of *T. gondii* to the health of southern brown bandicoot populations on the northern hinterland of Western Port, Victoria and methods of predicting probability of infection with environmental *T. gondii*.

A series of necropsies was performed on 33 southern brown bandicoots collected opportunistically over a five-year period. The causes of death were identified as motor vehicle trauma (22); predation (4); ejected pouch young (2); drowning (1); pyometra (1); possible toxicity and stomach bloating through lentil ingestion (1) and unknown (2). Real-time qPCR was performed on tissues from 30 cases, all of which were negative for the presence of *T. gondii* DNA. A range of helminths and ectoparasites were collected and identified, most of which had been previously been reported in this species. However, a *metastrongyloid* helminth species, found by histopathology in the lungs, is reported for the first time in a southern brown bandicoot.

To assess environmental contamination with *T. gondii*, two sites with different cat densities were compared. Seroprevalence of antibodies to *T. gondii* (n=24) using the Modified Agglutination Technique was performed on trapped southern brown bandicoots.

No evidence of infection with *T. gondii* was found at either site. Molecular (qPCR) methods were used to measure *T. gondii* oocyst presence in soil samples (n= 594) and prevalence of *T. gondii* in tissues of rabbits (n=118) and mice (n=267). All tests were negative across both sites except for the presence of *T. gondii* in one rabbit (prevalence 0.85%). These results suggest that rabbits and mice may have the potential to be reliable sentinel species and inform conservation management of the probability of infection with *T. gondii* in small marsupials.

The results from this study are consistent with the concept that opportunistic necropsy is a valuable strategy for passive disease and cause of death surveillance in native wildlife. No evidence was found that *T. gondii* was impacting the health of southern brown bandicoots, however, further longitudinal health surveys are necessary to determine the true prevalence of disease and causes of mortality. Further studies are recommended to confirm the effectiveness of mice and rabbits as potential sentinel species in a range of ecosystems, including those environments which have higher levels of contamination with *T. gondii*.

Declaration by Author

I certify that:

- i. this thesis comprises only my original work towards the degree of Masters of Veterinary Science, except where indicated in the preface and acknowledgement sections
- ii. due acknowledgement has been made in the text to all other material used
- iii. the thesis is fewer than 50,000 words in length, exclusive of maps, tables, bibliographies and appendices.

Amanda Jane Breidahl

Preface

Contribution of others:

Honorary Professor Ian Beveridge (Faculty of Veterinary and Agricultural Science, The University of Melbourne) identified all of the internal parasites, both gross specimens and histopathology sections (Chapter 2); assisted in identification of some of the external parasites (Chapters 2 and 3); examined the blood smear slides for haemoparasites (Chapter 3); photographed the histopathology slides in Plates 2.1 and 2.2 (Chapter 2) and proof read the sections in the Thesis referring to parasites (Chapters 2 and 3).

Dr David Spratt (Honorary Fellow in the Australian National Wildlife Collection, National Research Collections, Australia Commonwealth Scientific and Industrial Research Organisation) also examined the blood smear slides for the presence of haemoparasites (Chapter 3).

Dr Jasmin Hufschmid (Faculty of Veterinary and Agricultural Science, The University of Melbourne) assisted in the identification of the external parasites (Chapters 2 and 3).

Dr Pam Whiteley (Victorian Wildlife Health Surveillance, The University of Melbourne) performed the gross necropsy on seven bandicoots (408-16, 409-16, 410-16, 432-16, 433-16, 434-16 and 435-16) (Chapter 2).

Dr Janeen Samuel (retired veterinary pathologist) examined and interpreted all of the histopathology slides (Chapter 2).

Pat Statham (Department of Primary Industries, Parks, Water and Environments, Mt Pleasant Animal Health Laboratory, Launceston, Tasmania) performed the Modified Agglutination Tests on eight southern brown bandicoot blood samples stored on Whatman Filter paper.

Mr Barry Murphy (Murphy Landscape Consultancy) prepared the maps in Figures 1.2, 2.1, 2.2 and 3.1.

Dr Duncan Sutherland (Phillip Island Nature Parks) prepared the maps in Figure 3.2.

Mr Ian Caldwell supplied the majority of the rabbits.

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Glass Sculpture

Artists: Janet Lawrence with David Lancashire and Kate Cullity, 2013. In memory of *Kevin Taylor*, one of the landscape architects who designed the Australian Garden Dreaming at the Royal Botanic Gardens Cranbourne

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CHAPTER 1

INTRODUCTION

1.1 Decline of Australian wildlife

Australia's unique fauna is under pressure, with the extinction rate of native species accelerating alarmingly since European settlement in 1778 (Woinarski *et al.* 2015). In addition, abundance, range and diversity of persisting endemic flora and fauna have seen significant declines (Woinarski *et al.* 2015). The Australian Environmental Protection, Biodiversity and Conservation (EPBC) Act 1999 records 54 fauna species as extinct, of which 27 are mammals, representing one third of the total mammalian extinctions globally since the 1500's (Woinarski *et al.* 2015; Woinarski *et al.* 2018). Of those species remaining, a further ten mammals are listed as critically endangered, 37 as endangered and 60 as vulnerable (Threatened Species Scientific Committee 2016a). Ninety-one of the original 305 terrestrial mammals present in 1778 are now absent from at least half of the bioregions that they originally inhabited (Burbidge *et al.* 2008) with a further 21% threatened in their original bioregions (Woinarski *et al.* 2015). The majority of these are small to medium-sized mammals in a critical weight range (CWR) of 35 – 5500g, with the most vulnerable being those which nest and habit on the ground or surface, as opposed to those that burrow and use rocks (McKenzie *et al.* 2007).

Threatening processes for Australian mammals are attributed to multiple and often inter-related factors impacting ecosystems including climate change (Burbidge *et al.* 2008); degradation, fragmentation and loss of habitat (McKenzie *et al.* 2007); introduced exotic weeds (McKenzie *et al.* 2007); changed land use to pastoralism (McFarlane *et al.* 2013); changed land management systems such as altered fire regimes (McKenzie *et al.* 2007; Woinarski *et al.* 2015); competition and degradation by introduced herbivores (McKenzie *et al.* 2007; Woinarski *et al.* 2015); poisoning by introduced cane toads (*Rhinella marina*) (Woinarski *et al.* 2018); disease (Scheele *et al.* 2019) and predation by introduced feral animals such as cats (*Felis catus*) (Abbott 2008; Woinarski *et al.* 2015), red foxes (*Vulpes vulpes*) and wild dogs (*Canis lupis familiaris*) (Abbott 2011; Robley *et al.* 2014; Woinarski *et al.* 2015).

Cats, in particular, are considered one of the most significant and pervasive current causes of declines in Australian native species (Woinarski *et al.* 2015). Cats occupy more than 99.8% of Australia's land mass (Legge *et al.* 2017) and have been highlighted as a significant threat to birds, reptiles and small CWR mammals, with mammals forming the majority of their diet (Kutt and Kitchener 2012). The Australian feral cat population is estimated to fluctuate between 2.1 and 6.3 million, according to seasonal conditions (Legge *et al.* 2017) and is estimated to kill approximately 272 million birds through predation (Woinarski *et al.* 2017) and 466 million reptiles (Woinarski *et al.* 2018) annually, with figures varying widely, depending on local conditions. The number of mammals killed annually by cats is estimated to be 1.144 billion, of which 459 million are native species (Murphy *et al.* 2019), representing at least 151 different extant, terrestrial species (Woolley *et al.* 2019). In addition, the introduction of cats to Australia has resulted in the introduction of toxoplasmosis, a potentially fatal parasitic disease of warm-blooded vertebrates, caused by the protozoan *Toxoplasma gondii*, which has been shown to infect Australian mammal species (Canfield *et al.* 1990; Dubey 2010).

There have been several examples of other disease agents significantly impacting populations of Australian wildlife, including *Chlamydia* spp infections in the koala (*Phascolarctos cinereus*) (Burbidge *et al.* 2008); chytridiomycosis in amphibians (Berger *et al.* 2005; Scheele *et al.* 2019; Skerratt *et al.* 2010); and Devil Facial Tumour Disease (DFTD) in Tasmanian devils (*Sarcophilus harrisii*) (Hamede *et al.* 2013; Pye *et al.* 2016a; Pye *et al.* 2016b). Bandicoot species, such as the southern brown bandicoot (*Isodon obesulus obesulus*), represent some of the marsupials which are subject to several interacting threatening pressures including habitat loss, predation and disease (Woinarski *et al.* 2014b).

1.2 Southern brown bandicoot

The southern brown bandicoot is a small, dusky-brown coloured, omnivorous, crepuscular marsupial with a distinctive pointed snout (Fig 1.1) (Jackson 2003a).



Figure 1.1 Southern brown bandicoot (*Isoodon obesulus obesulus*) (photograph courtesy of Hayley Davis)

Its range has historically extended along the south-eastern Australian coastline from Sydney to Adelaide, including Tasmania, however, both range and abundance on the mainland have dramatically declined over recent decades (Brown and Main 2010; Coates *et al.* 2008). There is a strong argument that quenda (*Isoodon obesulus fusciventer*), currently considered a western sub-species of the southern brown bandicoot, may in fact be a separate species (Travouillon and Phillips 2018). For the purpose of this thesis, they are differentiated by their common names. The southern brown bandicoot is now listed as ‘endangered’ under the national EPBC Act 1999 (Threatened Species Scientific Committee 2016b), ‘threatened’ under the Victorian Flora and Fauna Guarantee (VFFG) Act 1988 (July 2016 list) (Department of Environment Land Water & Planning 2016), and as ‘near threatened’ in the Advisory List of Threatened Vertebrate Fauna in Victoria (Department of Sustainability and Environment 2013). It is regionally extinct in many parts of Victoria where it was once abundant (Coates *et al.* 2008; Menkhorst and Loyn 2011). However, remnant populations of southern brown bandicoots persist in south-central Victoria on the northern edge of Western Port and associated hinterland (Coates *et al.* 2008). Protected populations reside within reserves with predator exclosure fences, including the Royal Botanic Gardens Cranbourne (RBGC), approximately 70 km south-east of Melbourne, the Bayles Fauna Park and the Bayles Bandicoot Corner. Further protected populations persist on Quail and Chinaman Islands in Western Port (Bryant *et al.* 2018). Free-ranging populations exist outside the RBGC in the Cranbourne area, extending south

easterly encompassing an area formerly known as the Koo Wee Rup Swamp and the townships of Koo Wee Rup and Bayles (Atlas of Living Australia; Coates *et al.* 2008; Nicholls *et al.* 2018). Many live in close association with human habitation, peri-urban areas, farm lands and along railway corridors and roadside drains (Coates *et al.* 2008; Maclagan 2016; Maclagan *et al.* 2018; Nicholls *et al.* 2018) (see Figure 1.2).

The causes of decline in abundance and geographical distribution of the southern brown bandicoot are similar to those of other Australian mammals. Increasing urbanisation, concomitant with habitat loss and fragmentation have been significant factors (Coates *et al.* 2008). In addition, their small mass, falling within the CWR, along with ground foraging and ground nesting behaviour, makes the southern brown bandicoot ideal prey for cats, dogs and foxes (Abbott 2011; McKenzie *et al.* 2007; Woinarski *et al.* 2015). An Action Plan for the recovery of population numbers and environmental occupancy has been outlined with a number of approaches to mitigate threats (Woinarski *et al.* 2014a). Recommendations include predator control; habitat restoration and connectivity; covenanting private properties; weed management; appropriate cool-burn fire regimes; reducing road kill; increasing community engagement; implementing captive breeding and translocation programs and understanding the role disease may be playing in population decline (Woinarski *et al.* 2014a). Translocating animals from areas of poor habitat quality and high risk to areas of higher quality habitat with decreased predator presence and disease risk will help to establish insurance populations, as has occurred with the eastern barred bandicoot (*Perameles gunnii*) (Adriaanse 2018) (Sutherland, D., 2018. Pers comm.).

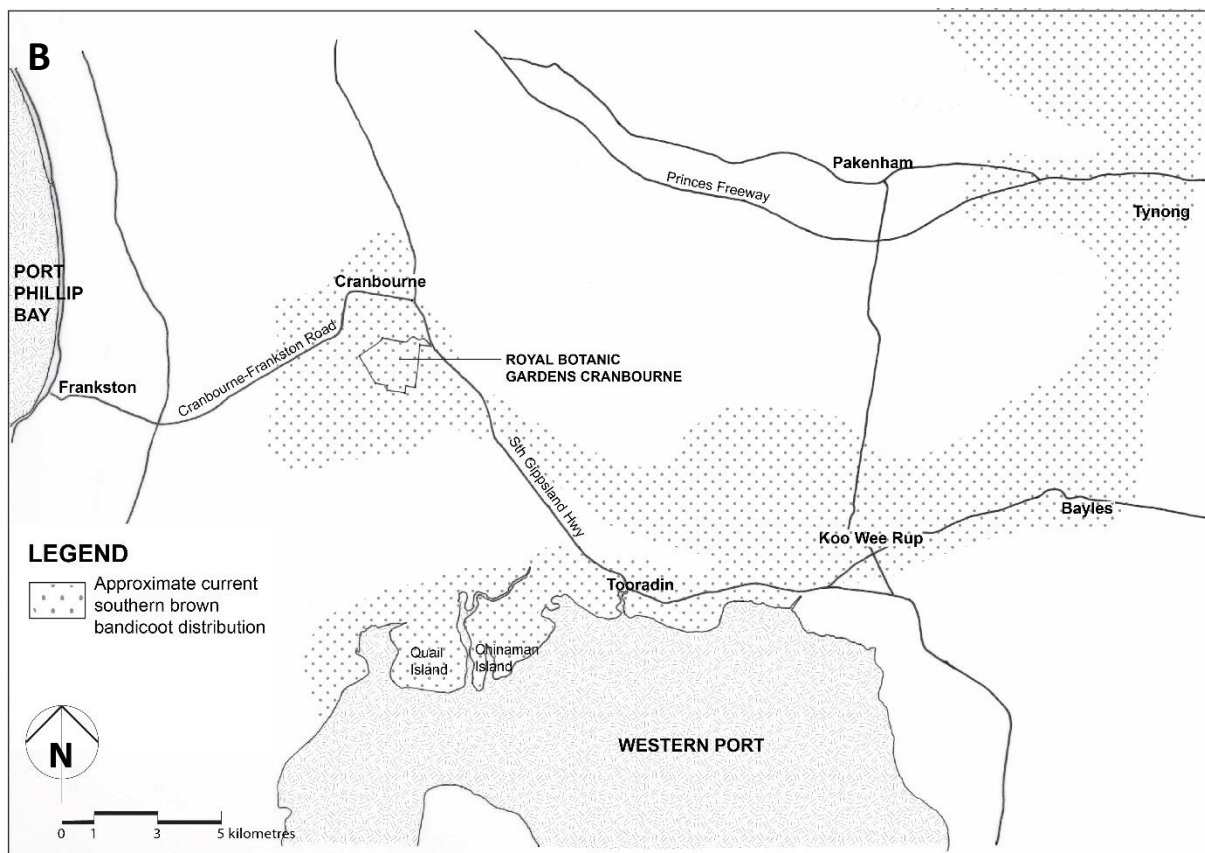
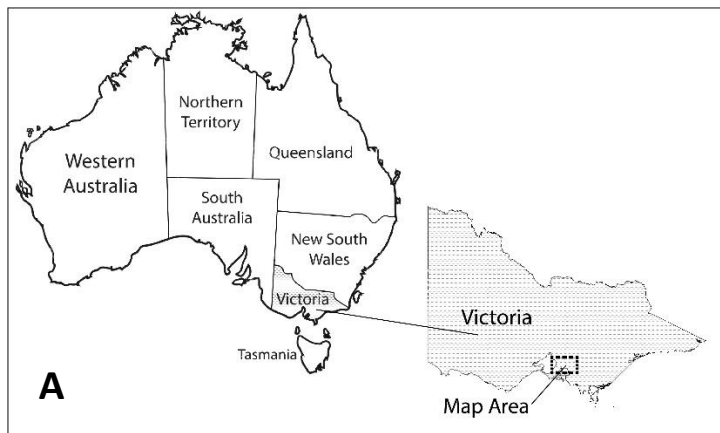


Figure 1.2 One of the remnant sites for free-ranging southern brown bandicoot (*Isodon obesulus obesulus*) populations on northern Western Port, Victoria, Australia (A) and approximate current distribution including Quail Island, Chinaman Island, Royal Botanic Gardens Cranbourne, Koo Wee Rup and Bayles (B).

Ecology

Adult southern brown bandicoots weigh from 400 to 1600 g (Jackson 2003a). Living between two to four years in the wild, they begin breeding from four months of age, using ground nests and produce several litters per year with a peak breeding season in

spring (Jackson 2003a). Litter size varies from one to six, averaging 2.5 young. Following a gestation period of around 12 days, young make their way to the pouch (Menkhorst and Seebeck 1995). Pouch emergence begins around 42 - 49 days (Duffy and Rose 2008), with full emergence and weaning between 60 and 70 days of age (Duffy and Rose 2008; Jackson 2003a).

Southern brown bandicoots rely on dense groundcover protection, preferring heathy, coastal woodland, from which they will venture forth to feed in more open country (Brown and Main 2010; Coates *et al.* 2008; Southwell *et al.* 2008). They have been shown to also live in close association with human dwellings and along roadside verges and drains in peri-urban areas (Maclagan 2016; Maclagan *et al.* 2018). Southern brown bandicoots forage through digging into the earth with their snouts, leaving distinctive conical shaped holes. They have a faunivorous diet consisting of invertebrates (including earthworms, arthropods and molluscs), seeds, grasses and a wide range of fungi (Jackson 2003a) (Maclagan, S. 2019. Pers. comm.). Each bandicoot is estimated to turn over up to 3.9 tons of soil per year (Valentine *et al.* 2012). In so doing, they perform an important ecological role aerating soil, improving soil moisture, enhancing seedling recruitment and distributing leaf litter (Valentine *et al.* 2017) resulting in improved plant health and biodiversity (Fleming *et al.* 2014).

Causes of death

Opportunistic necropsies and conservation management reports suggest that the most common known causes of death in Victorian southern brown bandicoots are motor vehicle trauma and predation (Coates, 2018 Pers. comm.) (Jackson 2003a; Jansen 2017; Robley *et al.* 2014). However, much about the health of these bandicoots remains unknown. Disease, in particular infection with *T. gondii*, may represent a further threatening process (Lenghaus *et al.* 1990b; Obendorf *et al.* 1996). As part of the overall conservation management of this species and as part of assessing risks associated with translocation programs, it is important to understand which diseases may be affecting southern brown bandicoots both at an individual and population level (Jakob-Hoff 2019).

Significant diseases of bandicoot species

Disease in the southern brown bandicoot has not been extensively documented. While there have been isolated necropsy reports in the national database (Lynch 2010; WHA 2007), most of our knowledge is inferred from the eastern barred bandicoot (*Perameles gunnii*), western barred bandicoot (*Perameles bougainville*), quenda and northern brown bandicoot (*Isodon macrourus*). Reported disease agents include ectoparasites, helminths, coccidia, haematozoa, viruses and secondary bacterial infections (see Table 1.1 for detail). Many are reported in captive animals and captivity may have played a role in their aetiology.

Parasites are considered part of the normal flora of the bandicoot (Clark *et al.* 2004; Roberts 1970; Spratt and Beveridge 2016). They are usually only of concern when present in very high numbers (Lynch 2010), which may occur when other factors are at play, decreasing host immunity. Parasitic infection may alter immune responses (Hing *et al.* 2016a) and heavy infections can cause clinical symptoms including anaemia, malnutrition, weakness and even death in the case of *T. gondii* (Bettioli *et al.* 2000a; Obendorf and Munday 1990).

Reports of ectoparasite (Gemmell *et al.* 1991; Lenghaus *et al.* 1990b) and endoparasite infestations in bandicoot species include a range of helminths (Spratt and Beveridge 2016); coccidia (*Eimeria* spp. (Lynch 2010); *Klossiella* spp (Bennett *et al.* 2007) and *Toxoplasma gondii* (Booth *et al.* 1995; Lenghaus *et al.* 1990b; Obendorf *et al.* 1996)); haematozoa (*Babesia* spp., *Theileria* spp. and *Hepatozoon* spp.) (Clark *et al.* 2004; Wicks and Clark 2005; Wicks *et al.* 2006); trypanosomes (Bettioli *et al.* 1998) and *Giardia* spp. (Bettioli *et al.* 1997). *Cryptosporidium* spp. have been shown to be shed in the faeces of southern brown bandicoots (prevalence of 12.2%) in the Sydney area (Dowle *et al.* 2013). However, while *Cryptosporidium* is a potential zoonosis, there has been no evidence to show that it causes disease in bandicoots (Dowle *et al.* 2013).

Primary bacterial infections appear to be rare, with reported bacterial infections having mostly occurred as secondary infections to trauma (Lynch 2010).

A number of bandicoot viruses have recently been described, including papilloma and herpesviruses. The diagnosis and identification of the novel virus, Bandicoot Papillomatosis Carcinomatosis Virus 1 (BPCV1) in the endangered western barred bandicoot (Bennett *et al.* 2008; O'Hara *et al.* 2004; Woolford *et al.* 2008) and Bandicoot Papillomatosis Carcinomatosis Virus 2 (BPCV2) in the quenda (Bender *et al.* 2019) raises concerns for these fragile populations. These contagious viruses cause debilitating papillomatous lesions on the face, eyes, mouth and feet of the bandicoot, which transform to carcinomatous lesions (O'Hara *et al.* 2004), often resulting in death or euthanasia (Woolford *et al.* 2008). The syndrome has occurred in Western Australia (WA) in captive, breeding colonies of western barred bandicoot and in the wild at Bernier Island, WA (Woolford *et al.* 2008). Onset occurs between one and five years of age (average 3 years 2 months) with death occurring between two weeks and 4.5 years (average 1 year and 4 months) after onset of skin lesions (Woolford *et al.* 2008). A herpes virus, Peramelid herpesvirus 1 (PeHV-1), has also been identified in quenda in WA, although not a lot is known of its pathogenicity or aetiology other than that it causes skin lesions with epithelial hyperplasia (Bender *et al.* 2019). Some of these new viruses may represent a serious risk to bandicoot populations, especially to the endangered western barred bandicoot. They have not yet been recorded in Victorian bandicoot species.

Other reported pathology in bandicoot species includes nephritis and pyelonephritis (Lynch 2010; WHA 2007); gastric ulceration (Lynch 2010; WHA 2007); chronic fibrosing pancreatitis (WHA 2007); ocular disease, including infection with *Chlamydia* spp. (Warren *et al.* 2005) and bacterial septicaemia secondary to wounds, such as *Pasteurella multocida* (Lynch 2010); *Vibrio* spp. (Lynch 2010) and *Erysipelothrix rhusiopathiae* (Eamens *et al.* 1988; Lynch 2010).

Table 1.1 Some reported pathogens and parasites responsible for disease in Australian bandicoot species: eastern barred bandicoot (*Perameles gunnii*); long-nosed bandicoot (*P. nasuta*); western barred bandicoot (*P. bougainville*); northern brown bandicoot (*Isodon macrourus*); southern brown bandicoot (*I. obesulus obesulus*) and quenda (*I. obesulus fusciventer*). *sub-species not specified

Pathogen or pathology	Bandicoot species	Author
Ectoparasites		
Ticks	<i>P. gunnii</i>	(Lenghaus <i>et al.</i> 1990b)
	<i>I. macrourus</i>	(Gemmell <i>et al.</i> 1991)
Endoparasites		
Helminths	all	(Lynch 2010; Spratt and Beveridge 2016)
Protozoa		
Coccidia, unidentified	<i>I. obesulus obesulus</i>	(Lynch 2010)
	<i>P. gunnii</i>	(Lynch 2010)
<i>Eimeria</i> spp.	<i>P. gunnii</i>	(Lynch 2010)
<i>Klossiella quimrensis</i>	<i>P. bougainville</i>	(Bennett <i>et al.</i> 2007)
	<i>I. obesulus obesulus</i>	(Jansen 2017)
	<i>P. gunnii</i>	(Lenghaus <i>et al.</i> 1990b)
		(Samuel, J. pers comm.)
<i>Toxoplasma gondii</i>	<i>P. gunnii</i>	(Bettiol <i>et al.</i> 2000a; Groenewegen <i>et al.</i> 2017; Lenghaus <i>et al.</i> 1990b; Obendorf <i>et al.</i> 1996)
		(Samuel, J. pers comm.)
	<i>I. obesulus fusciventer</i>	(Hillman <i>et al.</i> 2017b)

Pathogen or pathology	Bandicoot species	Author
<i>Sarcocystis</i> spp.	<i>P. gunnii</i>	(Obendorf and Munday 1990)
	<i>P. bougainville</i>	
	<i>P. nasuta</i>	(Lynch 2010)
Haematozoa		
<i>Babesia</i> spp.	<i>I. obesulus</i> *	(Clark <i>et al.</i> 2004)
<i>Theileria</i> spp.	<i>I. obesulus</i> *	(Clark <i>et al.</i> 2004)
	<i>I. macrourus</i>	(Clark <i>et al.</i> 2004)
	<i>P. nasuta</i>	(Clark <i>et al.</i> 2004)
<i>Hepatozoon</i> spp.	<i>P. gunnii</i>	(Clark <i>et al.</i> 2004)
	<i>I. obesulus obesulus</i>	(Wicks and Clark 2005)
<i>Trypanosoma</i> spp.	<i>P. gunnii</i>	(Bettioli <i>et al.</i> 1998)
<i>Giardia</i> spp.	<i>P. gunnii</i>	(Bettioli <i>et al.</i> 1997)
	<i>I. macrourus</i>	(Lynch 2010)
Viruses		
Peramelid herpesvirus 1 (PeHV-1)	<i>I. obesulus fusciventer</i>	(Bender <i>et al.</i> 2019)
Bandicoot Papillomatosis Carcinomatosis Virus 1 (BPCV1)	<i>P. bougainville</i>	(Bennett <i>et al.</i> 2008; O'Hara 2004; Woolford <i>et al.</i> 2008)
Bandicoot Papillomatosis Carcinomatosis Virus 2 (BPCV2)	<i>I. obesulus fusciventer</i>	(Bender <i>et al.</i> 2019)
Bacteria		

Pathogen or pathology	Bandicoot species	Author
<i>Erysipelothrix rhusiopathiae</i>	<i>I. macrourus</i>	(Eamens <i>et al.</i> 1988)
	<i>I. obesulus obesulus</i>	(Lynch 2010)
<i>Vibrio</i> spp.	<i>P. gunnii</i>	(Lynch 2010)
<i>Pasteurella multocida</i>	all	(Lynch 2010)
Other pathology		
Nephritis, pyelonephritis & nephrosis	all	(Lynch 2010)
	<i>P. gunnii</i>	(Booth <i>et al.</i> 1995)
Cholelithiasis	<i>P. gunnii</i>	(Lynch 2010)
Chronic pancreatitis	<i>P. gunnii</i>	(Lynch 2010)
	<i>I. obesulus obesulus</i>	(Lynch 2010)
Gastric ulceration	<i>P. gunnii</i>	(Lynch 2010)
	<i>I. obesulus obesulus</i>	(Lynch 2010)
Ocular disease (<i>Chlamydia</i> sp.)	<i>P. bougainville</i>	(Warren <i>et al.</i> 2005)

Toxoplasma gondii is a potentially significant pathogen of bandicoots which has been shown to be the cause of clinical disease and death in free-ranging eastern barred bandicoots (Booth *et al.* 1995; Lenghaus *et al.* 1990a; Obendorf and Munday 1990; Obendorf *et al.* 1996). However, the prevalence of infection with *T. gondii* in southern brown bandicoot populations and its impacts at the individual and population level remain largely unstudied.

1.3 *Toxoplasma gondii*

Life cycle and epidemiology

Toxoplasma gondii is an obligate, intracellular, protozoan parasite. It is distributed worldwide (apart from Antarctica) and is considered to be one of the most widespread

parasites of endothermic vertebrates (Lélu *et al.* 2013). While all endothermic vertebrates can act as intermediate hosts, the only known definitive hosts are felidae (Dubey 2010; Guo *et al.* 2015). After a cat ingests prey with infective tissue cysts containing bradyzoites, the cysts are broken down by the cat's digestive system and the released bradyzoites penetrate the intestinal epithelial cells, replicating asexually over many cycles in a process called merogony (Dubey 2010). A less common route of infection for cats is by ingesting tachyzoites in prey or directly ingesting sporulated oocysts from infective feline faeces in the environment (Dubey 2010; Lélu *et al.* 2010). Between 3 - 15 days after ingesting tissue cysts containing bradyzoites, gamonts (containing male or female gametes) are formed throughout the small intestine. Sexual fertilisation follows with the formation of walled zygotes within the epithelial cells, which then rupture and release oocysts into the intestinal lumen (Dubey 2010). Shedding occurs in nearly 100% of cats after ingesting tissue cysts containing bradyzoites (Dubey 2010) with oocysts appearing in their faeces approximately 3- 18 days after ingestion, regardless of the number of bradyzoites ingested (Dubey 2001). If cats ingest tachyzoites, then oocyst shedding occurs between 11 and 18 days later (Dubey 2010). Shedding of oocysts occurs a minimum of 18 days after ingestion of sporulated oocysts, regardless of infective dose (Dubey 2010). Approximately 1% of cats are shedding at any point in time (Dubey 2010) with some studies finding up to 13 million oocysts per gram of cat faeces (Dubey 2010). The number of oocysts shed does depend on the infective dose of bradyzoites and more than 187 million shed oocysts have been measured in faeces over 17 days from a cat fed 1,000 bradyzoites (Dubey 2001). The number of oocysts per gram of faeces in free-ranging, naturally infected cats is largely unknown with worldwide studies reporting between 0 and 66% of faeces examined containing oocysts (Dubey 2010).

Cats are mostly sub-clinical during initial infection, although in some cases clinical signs have been described, including fever, anorexia, diarrhoea, dyspnoea, uveitis and neurological symptoms, occasionally resulting in death (Dubey 2010; Enache *et al.* 2016). Oocyst shedding occurs for about one week in most cats (Dubey 2010). Following initial infection, immunity to re-shedding oocysts is developed, along with an antibody titre (Dubey 2010). However, re-infection and re-shedding can occur and is

thought to be related to immune status and stressors affecting the cat, including the cat's age and nutritional status, the infective dose and strain and possible concurrent infection with *Cystoisospora felis* (Dubey 2010) or Feline Immuno-deficiency Virus (Foster *et al.* 1998). Persistence of immunity to re-shedding of oocysts in the face of new infection varies, with immunity tending to decline over time in experimental infections (Dubey 1995a; Zulpo *et al.* 2018). It is not known if re-shedding occurs in naturally infected or free-ranging cats, nor the frequency (Dubey 1976; Dubey 2010; Jones and Dubey 2010). However, as cats are likely to be repeatedly exposed in natural situations, Zulpo (2018) suggests that they are also likely to be re-shedding and contaminating the environment at different periods throughout their life, despite maintaining high antibody titres.

Once in the environment, oocysts sporulate within one to five days and are then infective to an intermediate host (Dubey 2010). This period varies with aeration and temperature (Dubey 2010). Sporulated oocysts are more resistant than un-sporulated cysts (Dubey 2010). Desiccation, high temperature, low humidity and UV rays all decrease length of viability of oocysts in the environment (Dubey 1998; Dubey 2010). In natural conditions, oocyst survival is increased if the faeces are buried, and have been shown to survive for up to 18 months when buried outdoors in temperatures ranging from 20-35°C (Dubey 2010). These factors largely define the geographical locations where *T. gondii* infections are most prevalent (Cox 1981; Dickson 2018; Lélou *et al.* 2010).

Oocysts contaminate vegetation, soil and waterways. They are moved through the soil with rainwater, ultimately contaminating waterways, oceans and remote islands, becoming a source of infection for aquatic mammals and birds (Carlson-Bremer *et al.* 2015; Jones and Dubey 2010; Miller *et al.* 2008; Verant *et al.* 2014). Insects, such as flies and cockroaches, can move buried oocysts to the surface (Dubey 2010). Transport hosts such as earthworms, cockroaches and other invertebrates can ingest oocysts becoming a further source of infection for animals, such as bandicoots (Bettiol *et al.* 2000b). Migratory birds, such as the barnacle goose (*Branta leacopsis*) can also act as

vectors, transporting *T. gondii* to remote regions (Prestrud *et al.* 2007). Herbivorous intermediate hosts are generally infected through ingestion of vegetation or soil contaminated with oocysts, while carnivorous hosts are most commonly infected through ingestion of tissue cysts in infected prey species; both pathways result in the formation of tissue cysts (Dubey 2010).

After ingestion by the intermediate host, sporozoites are released from the sporulated oocyst and penetrate the intestinal wall, where within two hours they are found in enterocytes, goblet cells, the lamina propria, vascular tissue and white blood cells including mononuclear cells and segmented leukocytes (Dubey 2010). The sporozoites divide each into two tachyzoites within 12 hours and are transported in the vascular and lymphatic systems to local mesenteric lymph nodes and throughout the body (Dubey 2010). They penetrate tissue cells, where they are surrounded by a protective vacuole, form into tachyzoites (rapidly dividing forms) and undergo repeated asexual replication until the host cell ruptures (Dubey 2010). Within six days, the tachyzoites change into bradyzoites (slowly-dividing forms) and form localised, intracellular tissue cysts in a variety of organs including brain, spinal cord, choroid, liver, kidney, diaphragm, skeletal and cardiac muscular tissue where they can persist over long time periods (Dubey 2010). Tachyzoites gradually disappear from the visceral organs by three weeks post infection (Dubey 2010).

Most intermediate hosts survive initial infection and mount an immunological response, showing no or few clinical signs (Dubey 2010). Initial infection may cause intestinal and mesenteric lymph node necrosis alone or focal necrosis followed by inflammation (as a result of the rapid intracellular replication of tachyzoites) in a variety of organs, in particular eyes, heart, brain, skeletal muscle, lungs and adrenal glands (Dubey 2010). Extensive, multi-organ infection and associated inflammatory response can lead to acute clinical symptoms (toxoplasmosis) in some intermediate hosts, often resulting in death (Dubey 2010). Typical symptoms include lethargy and loss of appetite; respiratory distress; gastrointestinal symptoms (such as diarrhoea); neurological symptoms or sudden death (Canfield *et al.* 1990; Dubey 2010). Cysts in their dormant

phase typically do not cause clinical disease (known as latent toxoplasmosis), although there is evidence that they may be responsible for behavioural change of the intermediate host which may increase the risk of predation (Berdoy *et al.* 2000; House *et al.* 2011; Mahmoud *et al.* 2016; Vyas *et al.* 2007). Bradyzoites replicate slowly with occasional cyst rupture, maintaining host immunity (premonition) which in turn limits the pathogenicity of the parasite (Dubey 2010). If a host becomes immunocompromised, the asexual reproduction of the bradyzoites is no longer suppressed and clinical disease is re-activated (Dubey 2010). The reasons why an animal succumbs to initial infection or to recrudescent disease are unclear. However, factors such as host species and age, strain of *T. gondii*, stage of infective parasite at ingestion, infective dose and route, condition of host, stress factors, sex, concurrent infections, lactation, evolutionary factors, genetic makeup, ecological factors and immunological competence may all play a role (Dubey 2010).

Transplacental transmission is known to occur with blood-born tachyzoites crossing the placental barrier and infecting the foetus (Dubey 2010). *Toxoplasma gondii* has been shown experimentally to be transmitted through successive generations of mice (*Mus musculus*) in this manner without further re-infection with oocysts and often without developing antibodies (Dubey 2010). Vertical transmission has also been demonstrated in a variety of species, including humans (Dubey 2010); mice (Lélu *et al.* 2013); brown rats (*Rattus norvegicus*) (Dass *et al.* 2011); Californian sea-lions (*Zalophus californianus*) (Carlson-Bremer *et al.* 2015) and western grey kangaroos (*Macropus fuliginosus*) (Parameswaran *et al.* 2009b). This implies that infection within a population can persist without further exposure to environmental oocysts and may play a role in perpetuating the parasite in hostile environmental conditions (Pan *et al.* 2012). Venereal transmission of the parasite has been shown to occur in experimental brown rats (Dass *et al.* 2011). It is unknown whether vertical or venereal transmission occur in other marsupials or in free-ranging mice in Australia.

Diagnosis of Toxoplasmosis in animals

Antemortem diagnosis of toxoplasmosis is difficult. A clinical history, such as a marsupial experiencing some degree of stress in a captive situation with food potentially contaminated by cat faeces, combined with clinical signs is suggestive. The only definitive diagnosis is by demonstrating a 16-fold rising immunoglobulin (Ig) antibody titre between two blood samples taken 2 - 4 weeks apart using serological tests (Dubey 2010). This will only be useful if the blood samples are taken during the initial infection stage and not after the titre has reached its peak (Dubey 2010). Early infection may be detected if IgM antibodies are measured using the Direct Agglutination Technique (DAT) (Johnson *et al.* 1989). IgM antibodies begin circulating shortly post-infection but disappear within a few weeks (Hill and Dubey 2002). The most reliable, commonly used cross-species test is the Modified Agglutination Test (MAT) (Statham, P. 2018. Pers. comm.), however, it only measures IgG antibodies, which are not produced until two to three weeks post -infection (Bettioli *et al.* 2000a; Dubey 2010), hence may not detect early infections. The sensitivity of the kit is reported by the manufacturers to be 96.2% for human serum with a specificity of 98.8%. It has the advantage of being non-species specific and is regarded to translate well to marsupial species (Statham, P. 2018 Pers. comm.). Using Bayesian latent class analysis, Hillman (2017) calculated a specificity of 93% (79-99) for quenda but was unable to precisely calculate sensitivity. Sensitivity was similarly determined, using serum collected by cardiac puncture from dead feral cats, as 96.1% (95% credible interval 91.5-98.8); the specificity was lower at 82.0% (65.1 -93.3) (Adriaanse *et al.* 2020).

Other, less commonly used serological tests include: enzyme-linked immunosorbent assay (ELISA), the indirect immunofluorescence technique (IFAT), the indirect Haemagglutination test (IHA), the Sabin-Feldman dye test and the latex agglutination test (LAT) (Hill and Dubey 2002; Wyrosdick and Schaefer 2015). The disadvantage of these tests is that they require species specific antibodies and there is little research available that has established sensitivity and specificity in each species.

Biopsy of tissues, for example skeletal muscle, lymph nodes or cerebro-spinal fluid can also give an antemortem diagnosis by identifying the characteristic tachyzoites and associated inflammation through impression smears stained with Giemsa, or through histopathology (using Haematoxylin and Eosin, Giemsa or Leishman stains) or immunohistochemistry (Dubey 2010). However, biopsies are difficult to obtain from wild animals, requiring capture and general anaesthesia. They also lack sensitivity as diagnosis relies on the samples biopsied containing the organisms.

Post mortem diagnosis of active toxoplasmosis can be made through a combination of gross pathological changes observed at necropsy and microscopic identification of tachyzoites and associated inflammation in tissue sections, using the above staining techniques (Dubey 2010). At high magnification and resolution, *T. gondii* can be differentiated from other coccidian parasites, through its particular morphological shape and dimensions (Dubey 2010). Examination of several tissue samples (including from the brain, spinal cord, chorio-retina, heart, lung, skeletal muscle, kidney, adrenal gland) increases the likelihood of identifying tachyzoites. Identification of bradyzoites in cyst formations, will confirm a past or latent infection, although bradyzoites may also be present in active toxoplasmosis as they begin to appear within six day of infection and tachyzoites take up to three weeks to disappear (Dubey 2010). While the use of immunohistochemistry does increase the sensitivity of histopathology over using haematoxylin and eosin stains alone, diagnosis using histopathology still has lower sensitivity and specificity than molecular methods, as it relies upon organisms being present in the section of the specimen that is selected and on visualisation and correct identification of those organisms.

Molecular methods, using polymerase chain reaction (PCR), have a high sensitivity and specificity for identifying the presence of *T. gondii* DNA (Belaz *et al.* 2015) and have typically targeted the B1 gene, which is repeated 35 times (Burg *et al.* 1989). However, another DNA fragment of the *T. gondii* genome containing 529 base pairs (529bp), that is repeated 200-300 times, has been used more recently, providing a significantly more sensitive target (Suviriyapaisa *et al.* 2017; Wyrosdick and Schaefer 2015; Homan *et al.* 2000). Recent Bayesian analysis of real time PCR testing for *T. gondii* DNA using the

529bp target, in feline tissues calculated a diagnostic sensitivity of 90.1% and specificity of 96.0% (Adriaanse *et al.* 2020). PCR can be undertaken following DNA extraction from a range of digested tissues including central nervous system (CNS), skeletal muscle, heart muscle, liver, kidney, lymph nodes and lung. However, unlike histopathology, PCR cannot differentiate between tachyzoites and bradyzoites and hence between an active, latent or recrudescent infection. It also relies on the organism being present in the tissues sampled (Wyrosdick and Schaefer 2015) (see Chapter Two for further description of qPCR).

Post mortem diagnosis can also be achieved using mouse bio-assay to isolate viable *T. gondii* (Dubey *et al.* 2015), however, this requires a laboratory set up with specific pathogen-free mice and specialist equipment.

Toxoplasma gondii in wildlife worldwide

Toxoplasma gondii is present on every continent across the world, other than Antarctica, with prevalence studies for antibodies to *T. gondii* showing that it can infect a wide range of species, including herbivores, such as the white-tailed deer (*Odocoileus virginianus*) (58.8% of tested animals) (Ballash *et al.* 2015); omnivores, such as the wild boar (*Sus scrota*) (14.7%) (Calero-Bernal *et al.* 2015) and the black bear (*Ursus americanus*) (84.2%) (Dubey *et al.* 2015); and carnivores, such as the red fox (51.2% and 38%, depending on study) (Calero-Bernal *et al.* 2015; Jakubek *et al.* 2001).

Prevalence of infection (as measured by seroprevalence of antibodies to *T. gondii* or prevalence of *T. gondii* DNA in tissues) tends to be greater in higher order predators, which rarely experience clinical disease (Dubey 2010), exemplified by raptor species (Aubert *et al.* 2008; Love *et al.* 2016); feline species, such as the Eurasian lynx (*Lynx lynx*) (Jokelainen *et al.* 2013); canids (Sobrino *et al.* 2007) and eastern quoll (*Dasyurus viverrinus*) (Fancourt *et al.* 2014). Prevalence has been shown to increase with proximity to human habitation and with cat density (Jokelainen *et al.* 2011; Lélou *et al.* 2010).

Clinical toxoplasmosis has been reported in a variety of wildlife, including terrestrial and aquatic mammals and birds. A range of clinical signs have been observed including generalised illness, lethargy, inappetence, abortion, neurological symptoms including changed behaviour patterns, pulmonary distress, gastro-intestinal symptoms, and death (Dubey 2010; Jokelainen and Nylund 2012; Jokelainen and Vikoren 2014). Some species tend to be more susceptible to developing clinical toxoplasmosis compared with others, as is the case with the European brown hare (*Lepus europaeus*) and the mountain hare (*Lepus timidus*) (Jokelainen *et al.* 2011). With the spread of the domestic cat across the Australian landscape over the last 240 years (Abbott 2008; Legge *et al.* 2017) and the consequent shedding of oocysts into the environment, Australian wildlife is now also exposed to infection with *T. gondii*.

***Toxoplasma gondii* in Australian wildlife**

Toxoplasma gondii, is known to infect both free-ranging and captive populations of a wide range of Australian mammals. Whilst many individuals may only experience sub-clinical infection and then mount an immune-response (Dubey 2010; Pan *et al.* 2012), clinical toxoplasmosis resulting in illness or death has been reported in many species (see Table 1.2). Marsupials are considered highly susceptible to clinical disease following infection (Canfield *et al.* 1990; Donahoe *et al.* 2015; Dubey 2010; Parameswaran *et al.* 2010). This may be explained by the fact that *T. gondii* is an alien parasite which only arrived after European settlement and, due to the absence of any native feline species, has not co-evolved with Australian fauna (Canfield *et al.* 1990; Dubey 2010; Rideout 2019). Another possible factor is that the metatherian immune response differs to that of eutherian species (Dubey 2010; Jurd 1994; Miller 2010) and may not be as well equipped to suppress initial infection and later, clinical disease.

It is likely that several further factors, including genotype and level of exposure (Dubey 2010; Parameswaran *et al.* 2010) play a role in the impact of infection with *T. gondii* in Australian wildlife. The probability of infection with *T. gondii* will be determined by free-ranging cat density, prevalence of infection in cats, oocyst shedding rates, environmental conditions favourable for the survival of sporulated oocysts and

pathway of exposure (e.g. ingestion of contaminated vegetation vs tissue cysts) (Dubey 2010; Fancourt and Jackson 2014; Fancourt *et al.* 2014). Feral cat density is, in turn, determined by complex ecological factors, including availability of food resources and presence and density of apex predators such as dingos, foxes and Tasmanian devils (Harris 2011; Hollings *et al.* 2013). The susceptibility of free-ranging Australian mammal populations to clinical toxoplasmosis may be influenced by environmental conditions causing decline in immunological competence such as prolonged drought, resulting in nutritional stress and increased predator pressure (Dubey 2010; Hollings *et al.* 2013).

The feeding behaviour of intermediate hosts may also affect the likelihood of infection, with those animals grazing or foraging close to the ground more likely to become infected compared with arboreal folivores. This is evidenced by the lack of reports of infection with *T. gondii* in free-ranging koalas (Taggart, 2019), which spend little time feeding on the ground (Marsh *et al.* 2013). In contrast, one study found a 6.3% seroprevalence in free-ranging brush tailed possums, which as well as being arboreal folivores, also spend significant time feeding on the ground (Eymann *et al.* 2006). Additionally, the structure of the environment and its proximity to urbanisation may influence prevalence of infection. As well as higher cat densities, urban areas may also provide damper soils, through watered gardens and lawns, favouring oocyst survival (Dubey, 2010). Hillman (2017) showed a higher prevalence of infection in quenda from urban compared with natural bush environments and Reiss (2015) also demonstrated a higher prevalence of infection with *T. gondii* in northern brown bandicoots (*Isodon macrourus*) in urban areas. It is possible that proximity to roads may result in damper soils, where the water drains from the roads, enhancing oocyst survival. This may account for an increased seroprevalence among animals killed on roads compared with culled animals (Hollings *et al.* 2013).

Many species have adaptive behaviours to minimise parasite ingestion, such as avoiding grazing close to droppings (Weinstein *et al.* 2018), which may act to also minimise the risk of ingestion of *T. gondii* oocysts. However, it is unclear whether such behaviours

have been specifically selected for by Australian mammal in the case of *T. gondii*, given the parasite is a relatively recent arrival in the Australian landscape.

There are a range of genotypes of *T. gondii* in wildlife worldwide (Dubey *et al.* 2015; Verin *et al.* 2013). The study and nomenclature of genotypes has expanded rapidly over the last two decades with typing determined by a variety of methods from the DNA variations at the SAG2 locus (Howe *et al.* 1997) to the use of *Toxoplasma* genome database (ToxoDB) PCR-restriction fragment length polymorphism (PCR-RFLP) (Amouei *et al.* 2020). Three main genotypes have been associated with wildlife infections, Types I, II and III, which are closely related clonal lineages (Dubey *et al.* 2015; Verin *et al.* 2013). These all have a common ancestral Type II parent and show little variation within each Type (Khan *et al.* 2011). In Europe, Type II is the most common genotype (Howe *et al.* 1997), while Type I is not present and Type III has a frequency in cats of only 1.8% (Amouei *et al.* 2020). Types I, II and III are all present in North America, although there have been at least 33 different genotypes identified in cats (Amouei *et al.* 2020). Type II is the most common associated with human infections in North America (Howe *et al.* 1997). More recently, a distinct Type 12, showing greater genetic diversity, has been identified from wild animals in North America but not yet from Europe (Khan *et al.* 2011). Type 12 is now the most common genotype found in cats in North America (Amouei *et al.* 2020). By contrast, South America, in addition to Types I, II and III, has many additional, much more widely diverse and genetically distinct genotypes, probably reflecting a separate evolutionary process, wider diversity of intermediate hosts and greater sexual reproduction in the definitive host (Lehmann *et al.* 2006). In Australian mammals, a wide range of genetic diversity of *T. gondii* DNA has been identified (Pan *et al.* 2012; Parameswaran *et al.* 2010). As well as the traditional Types I, II and III, atypical Type II- like genotypes are present and are thought to have evolved as a result of Australia's relative isolation (Parameswaran *et al.* 2010). These are now regarded as indigenous strains (Parameswaran *et al.* 2010). A number of genotypes have been found in marsupials, many of them atypical Type II-like, exemplified by one study which found 45 different genotypes in 16 free-ranging macropods, most of which were multiply infected (Pan *et al.* 2012). In another study, 67% (n=12) of PCR-positive marsupials had atypical Type II-like genotypes

(Parameswaran *et al.* 2010). The remaining positive samples were typical I, II and III genotypes. Atypical Type II-like genotypes have also been isolated from a long-nosed fur seal (*Arctocephalus forsteri*) (Donahoe *et al.* 2014), in free-ranging wombats (*Vombatus ursinus*) (Donahoe *et al.* 2015) and in a southern brown bandicoot (Jansen 2017).

In captive Australian mammals, other factors may affect the probability of toxoplasmosis, including low immunity to the parasite as a result of lack of prior exposure to it, high levels of the parasite in the captive environment and lowered host immunity due to stress of capture and captivity (Brown *et al.* 2005). Sources of infection with *T. gondii* in captive environments are from contamination of pasture, bedding and food with oocysts from cat faeces, or from being fed raw meat, containing bradyzoite cysts, in the case of dasyurids (Attwood *et al.* 1975). Fatal toxoplasmosis has been recorded in experimental infections of marsupials, including fat-tailed dunnarts (*Sminthopsis crassicaudata*) (Hufschmid pers comm), eastern barred bandicoots (*Perameles gunnii*) (Bettioli *et al.* 2000a) and macropods (Johnson *et al.* 1989; Lynch *et al.* 1993a) (Table 1.2).

Susceptibility to developing disease appears to vary with marsupial species. Carnivorous dasyurids, such as the eastern quoll, have been shown to be less likely to develop acute disease despite seroprevalence of up to 100%, with no cases of clinical toxoplasmosis diagnosed in these species (Fancourt *et al.* 2014; Hollings *et al.* 2013). Macropods, such as *Macropus rufus*, *M. fuliginosus*, and *M. robustus*, have been shown to be able to recover from acute infection and mount an antibody response (Pan *et al.* 2012; Parameswaran *et al.* 2009a). The severity of toxoplasmosis in marsupials varies from sub clinical, to mild, to acute disease and sudden death (Canfield *et al.* 1990; Dubey 2010). Acute clinical signs include lethargy and inappetence (Canfield *et al.* 1990; Donahoe *et al.* 2015; Sangster *et al.* 2012; Skerratt *et al.* 1997); respiratory signs, including dyspnoea and coughing (Canfield *et al.* 1990; Hartley 2006; Sangster *et al.* 2012); gastro-intestinal signs including diarrhoea (Canfield *et al.* 1990; Dubey 2010); disruption of vision with central blindness (Canfield *et al.* 1990; Donahoe *et al.* 2015);

clouding of the eye (Attwood *et al.* 1975); neurological signs including convulsions, incoordination (Attwood *et al.* 1975; Canfield *et al.* 1990; Donahoe *et al.* 2015), head tilt, circling (Donahoe *et al.* 2015), change in mentation, ataxia and paresis (Canfield *et al.* 1990; Donahoe *et al.* 2015; Sangster *et al.* 2012; Skerratt *et al.* 1997) and sudden death (Canfield *et al.* 1990; Hartley 2006; Hartley *et al.* 1990). Changed behaviour following infection with *T. gondii* has been demonstrated experimentally in rats and mice (House *et al.* 2011; Mahmoud *et al.* 2016; Vyas *et al.* 2007). It is possible that infection with *T. gondii* also causes behavioural change in Australian wildlife, potentially leading to death by predation or misadventure. Hollings (2013) suggests infection with *T. gondii* may affect risk-taking behaviour following the finding that seroprevalence of *T. gondii* antibodies is significantly higher in road-kill Tasmanian pademelons (*Thylogale billardierii*) as compared with those that have been culled (presumably representing a random sample of the population). However, while Hollings (2013) does identify a correlation between seroprevalence to *T. gondii* antibodies and roadkill, it was beyond the scope of that study to demonstrate whether or not infection with *T. gondii* definitively resulted in behavioural changes in pademelons, or whether those changes per se were the cause of a higher road mortality. Neither did the study explore whether other factors may be responsible for higher prevalence of infection in road-kill animals, such as the possibility that grazing areas beside roads may be damper and hence more favourable to oocyst survival.

Gross necropsy findings associated with toxoplasmosis in Australian mammals are varied, often with no or few discernible lesions. A range of organs can be infected including brain, spinal-cord, eyes, lungs, heart, liver, spleen, kidney, intestines, adrenal glands, skeletal muscle, pancreas, oesophagus, gastrointestinal tract, peritoneum and lymph nodes grossly demonstrating reddening, enlargement, congestion, oedema and haemorrhage (Canfield *et al.* 1990). Histopathological changes reflect inflammation with perivascular cuffing, granulomatous inflammation and focal necrosis in association with evidence of tachyzoites within cells and intracellular thin walled cysts containing bradyzoites (Attwood *et al.* 1975; Canfield *et al.* 1990; Dubey 2010). While Attwood *et al.* (1975) describe the presence of ‘cerebellar granuloma’ and ‘scattered granulomata’ in association with severe meningo-encephalomyelitis, these changes would currently

more accurately be described as granulomatous inflammation. Free zoites are often seen in interstitial tissue surrounding areas of focal necrosis (Canfield *et al.* 1990). Severe diffuse changes were seen histologically in fat-tailed dunnarts experimentally infected with TgDgAu1 strain Type II lineage *T. gondii*, including necropurulent, fibrinous myocarditis; multifocal necrotising cholangiohepatitis and diffuse lymphoplasmacytic interstitial nephritis (Hufschmid pers comm).

Many Australian mammals recover from initial infection and mount an antibody response, although the exact rate of this occurring is unknown and is likely to vary between species. Seroprevalence based on antibodies to *T. gondii* has been measured in free-ranging populations using different diagnostic techniques, with results ranging from 0% to 100% (see Table 1.2). The MAT (Ardiaca *et al.* 2016), as described by Dubey (2010), is currently the most commonly used test for detecting IgG antibodies to *T. gondii* in Australian wildlife as the antibodies tend to remain circulating over a long time-frame and the test is non-species specific (Wyrosdick and Schaefer 2015). The MAT has been validated in a range of marsupial species (Hillman *et al.* 2017a; Johnson *et al.* 1989; Parameswaran *et al.* 2009a) and is considered to have a consistently high sensitivity and specificity in Australian mammals (Statham, P. 2018. Pers comm.). Most studies found no relationship with sex but one study on western grey kangaroos near Perth, WA found a higher seroprevalence in females compared with males (Parameswaran *et al.* 2009a). Seroprevalence has been demonstrated to increase with age (Eymann *et al.* 2006; Fancourt *et al.* 2014; Miller *et al.* 2000) and is higher in carnivorous species (Hollings *et al.* 2013), as well as in animals living in cooler and wetter environments (Fancourt *et al.* 2014; Hollings *et al.* 2013; Jakob-Hoff and Dunsmore 1983). Hollings (2013) was not able to determine the population effects of increased exposure to *T. gondii* on pademelons, whilst Fancourt (2014) concluded that declining eastern quoll populations were probably not a result of *T. gondii* infection but due to increased predation by cats and competition for food resources and habitat.

Hillman (2016) challenges assertions that free-ranging marsupial populations are particularly susceptible to developing clinical toxoplasmosis and that *T. gondii* therefore

threatens population viability, arguing that few studies actually set out specifically to address these hypotheses. Hillman (2016) suggests that many studies had inadequate sample size, possessed inherent sampling bias and used poor diagnostic methods, citing papers written in the 1950s, 1960s and 1970s, which used mouse assay and histopathology as examples of diagnostic techniques with low sensitivity and specificity (Wyrosdick and Schaefer 2015). However, recent research using diagnostic techniques with much higher sensitivity and specificity, such as MAT and PCR, report ongoing evidence of infection with *T. gondii* and clinical toxoplasmosis in marsupials (Donahoe *et al.* 2015; Eymann *et al.* 2006; Fancourt *et al.* 2014; Groenewegen *et al.* 2017). Hillman (2016) additionally argues that it is difficult to draw conclusions about population effects of *T. gondii* infection from seroprevalence studies alone, as such studies will not reflect animals that may have died acutely from the disease or animals that may have early infection and not yet mounted an IgG antibody response, nor will they reflect the percentage of animals that develop clinical toxoplasmosis. This is a valid comment, especially in singular cross-sectional studies.

As endangered bandicoot species appear to be among those mammals which have been shown to develop clinical toxoplasmosis in free-ranging, captive and experimental situations (Table 1.2), further investigation is warranted to gain a better understanding of the likely level of exposure to *T. gondii* in free-ranging populations and the prevalence of infection, clinical disease and mortality.

Table 1.2 Reports of clinical toxoplasmosis in Australian mammals (Captive -C, Free-living -F, Unknown – U, Aquatic -A and Experimental -E) and studies of seroprevalence of antibodies to *Toxoplasma gondii* in free ranging mammals

Common name	Scientific name	Origin	Seroprevalence (%)	Year of study	Author
MONOTREMES					
Short-beaked echidna	Tachyglossus aculeatus	C		1986	(McOrist and Smales 1986)
		F		1986	(McOrist and Smales 1986)
VOMBATIFORMES					
Koala	Phascolarctos cinereus	C		2016	(Dubey et al. 1991)
		U		1990	(Canfield et al. 1990)
		C		1990	(Hartley et al. 1990)

Common name	Scientific name	Origin	Seroprevalence (%)	Year of study	Author
Bare-nosed wombat	<i>Vombatus ursinus</i>	U		1990	(Canfield <i>et al.</i> 1990)
		C		1997	(Skerratt <i>et al.</i> 1997)
		F		2015	(Donahoe <i>et al.</i> 2015)
		C		2006	(Hartley 2006)
		F	26.1	2005	(Hartley and English 2005)
MACROPODS					
Macropods	Not specified	U		1990	(Canfield <i>et al.</i> 1990)
Bennett’s wallaby	<i>Macropus rufogriseus</i>	C		2008	(Dubey and Crutchley 2008)
		F	8	2013	(Hollings <i>et al.</i> 2013)

Common name	Scientific name	Origin	Seroprevalence (%)	Year of study	Author
Eastern grey kangaroo	<i>Macropus giganteus</i>	E		1989	(Johnson <i>et al.</i> 1989)
Pademelon	<i>Thylogale billardierii</i>	F	45	1972	(Munday 1972)
		F	7-19	2013	(Hollings <i>et al.</i> 2013)
Tamar wallaby	<i>Macropus eugenii</i>	C		2008	(Dubey and Crutchley 2008)
		E		1993	(Lynch <i>et al.</i> 1993b)
		F	76	2019	(Taggart 2019)
Western grey kangaroo	<i>Macropus fuliginosus</i>	F	15.5	2009	(Parameswaran <i>et al.</i> 2009a)
		F	20	2019	(Taggart 2019)

Common name	Scientific name	Origin	Seroprevalence (%)	Year of study	Author
POSSUMS					
Possums	Not specified	U		1990	(Canfield <i>et al.</i> 1990)
Common brushtail possum	<i>Trichosurus vulpecula</i>	F	6.3	2006	(Eymann <i>et al.</i> 2006)
MEGACHIROPTERA					
Little red flying fox	<i>Pteropus scapulatus</i>	C		2012	(Sangster <i>et al.</i> 2012)
Spectacled flying fox	<i>Pteropus conspicillatus</i>	C		2012	(Sangster <i>et al.</i> 2012)
DASYURIDS					
Dasyurids	Not specified	U		1990	(Canfield <i>et al.</i> 1990)
Crest-tailed mulgara	<i>Dasyercus cristicauda</i>	E		1975	(Attwood <i>et al.</i> 1975)

Common name	Scientific name	Origin	Seroprevalence (%)	Year of study	Author
Dibbler	<i>Antechinus apicalis</i>	E		1975	(Attwood <i>et al.</i> 1975)
Eastern quoll	<i>Dasyuris viverrinus</i>	F	9.4 -100	2014	(Fancourt <i>et al.</i> 2014)
		F	58	2013	(Hollings <i>et al.</i> 2013)
Fat-tailed pseudo-antechinus	<i>Antechinus macdonnellensis</i>	E		1975	(Attwood <i>et al.</i> 1975)
Fat-tailed dunnart	<i>Sminthopsis crassicaudata</i>	E		1975	(Attwood <i>et al.</i> 1975)
		E		2018	(Hufschmid <i>Pers. comm</i>)
Kowari (brush-tailed marsupial rat)	<i>Dasyuroides byrnei</i>	F		1975	(Attwood <i>et al.</i> 1975)
		E		1975	(Attwood <i>et al.</i> 1975)
Numbat	<i>Myrmecobius fasciatus</i>	F	0	1983	(Jakob-Hoff and Dunsmore 1983)

Common name	Scientific name	Origin	Seroprevalence (%)	Year of study	Author
Spotted-tail quoll	<i>Dasyuris maculatus</i>	F	71	2013	(Hollings <i>et al.</i> 2013)
Tasmanian devil	<i>Sarcophilus harrisii</i>	F	33	2013	(Hollings <i>et al.</i> 2013)
BANDICOOTS and BILBYS					
Bandicoots	Not Specified	U		1990	(Canfield <i>et al.</i> 1990)
Bilby	<i>Macrotis lagotis</i>	U		1990	(Canfield <i>et al.</i> 1990)
Eastern barred bandicoot	<i>Perameles gunnii</i>	C and F	9-33	2000	(Miller <i>et al.</i> 2000)
		C		1995	(Booth <i>et al.</i> 1995)
		F	6.7	1996	(Obendorf <i>et al.</i> 1996)
		F		2017	(Groenewegen <i>et al.</i> 2017)
		E		2000	(Bettioli <i>et al.</i> 2000a)

Common name	Scientific name	Origin	Seroprevalence (%)	Year of study	Author
Northern brown bandicoot	<i>Isoodon macrouris</i>	F	22.2	2015	(Reiss <i>et al.</i> 2015)
		F	39 -50	1957	(Cook and Pope 1959; Pope <i>et al.</i> 1957 b; Pope <i>et al.</i> 1957 a)
Southern brown bandicoot	<i>Isoodon obesulus obesulus</i>	F	3.45	2017	(Jansen 2017)
		F	0	2019	(Taggart 2019)
Quenda	<i>Isoodon obesulus fusciventer</i>	F	2.9-3.7	2017	(Hillman <i>et al.</i> 2017b)
RODENTS					
Bush Rat	<i>Rattus fuscipes</i>	F	2	2019	(Taggart 2019)
CANIDS					
Dingo	<i>Canis familiaris dingo</i>	F	10	1990	(Johnson <i>et al.</i> 1990)

Common name	Scientific name	Origin	Seroprevalence (%)	Year of study	Author
AQUATIC MAMMALS					
Dugong	<i>Dugong dugong</i>	F A		2012	(Owen <i>et al.</i> 2012)
Indo-Pacific humpbacked dolphin	<i>Sousa chinensis</i>	F A		2003	(Bowater <i>et al.</i> 2003)
Long-nosed fur seal	<i>Arctocephalus forsteri</i>	F A		2014	(Donahoe <i>et al.</i> 2014)

Prevalence and effects of Toxoplasma gondii on bandicoot species

Toxoplasma gondii has long been identified as a potential concern for management of bandicoot species, with studies conducted as far back as 1955 (Cook and Pope 1959; Pope *et al.* 1957 b; Pope *et al.* 1957 a). The feeding behaviour of bandicoots increases their likelihood of ingesting infective, sporulated *T. gondii* oocysts as they feed on vegetation close to the ground and are constantly turning over soil ingesting arthropods, seeds and fungi (Maclagan, S. 2019. Pers. comm.). Clinical symptoms of toxoplasmosis in bandicoots are similar to those observed in other marsupials. Eastern barred bandicoots experimentally infected with *T. gondii* showed symptoms such as docility, incoordination, blindness, behaviour changes and eventual death 15 – 17 days post infection (Bettioli *et al.* 2000a). Free-ranging eastern barred bandicoots with toxoplasmosis exhibit docility, weakness, erratic movements, loss of balance and death (Obendorf *et al.* 1996). Toxoplasmosis was found to be associated with consistent weight loss and was the cause of death of at least two eastern barred bandicoots in a trial release at French Island, Victoria (Groenewegen *et al.* 2017). There are several other reports of clinical toxoplasmosis in free-ranging eastern barred bandicoots leading to death (Samuel, J. 2018, Pers. comm.) (Booth *et al.* 1995; Lenghaus *et al.* 1990b; Obendorf and Munday 1990; Obendorf *et al.* 1996). Ocular changes have been postulated to be responsible for some changed behaviour and increased chance of predation or road trauma (Miller *et al.* 1993).

The ability to determine the true prevalence and impact of *T. gondii* in free-ranging bandicoot populations is limited by several factors, including the probability of obtaining a truly random sample of dead or live individuals; difficulties in obtaining longitudinal data on individuals, even with the use of telemetry (Groenewegen *et al.* 2017) (Sullivan, D. 2019, Pers. comm.) and limitations on test specificity and sensitivity (Hillman *et al.* 2016). The identification and diagnosis of infected, live, free-ranging animals is also compromised, as infected animals may be in hiding, less likely to enter a trap or be a victim of predation (Wobeser 2007). Additionally, as discussed earlier, MAT will only detect the immune response of individuals which have survived initial

infection and have begun producing IgG antibodies. Bettiol (2000) found that the two eastern barred bandicoots experimentally inoculated with *T. gondii* oocysts developed IgM between 6 and 17 days post-infection and there was no detectable IgG by 17 days post infection. Tamar wallabies (*Macropus eugenii*) were shown to develop IgM antibodies between 7 and 12 days post experimental vaccination (Lynch *et al.* 1993a), while IgG has been shown to peak by three weeks post experimental infection in eastern grey kangaroos (*Macropus giganteus*) (Johnson *et al.* 1989). The timing for IgG antibody production in southern brown bandicoots has not been ascertained. The DAT will detect IgM however, it has not been well validated for marsupial species (Hillman *et al.* 2017a; Hillman *et al.* 2016) and anecdotal evidence suggests that it is not very specific in the detection of IgM against *T. gondii* in marsupial species (Statham, P. 2017. Pers comm.).

Despite these caveats, seroprevalence studies still provide good cross-sectional information. There have only been a handful of seroprevalence studies performed in bandicoots with results varying between 0 - 22.2% depending on species and geographic location (see Table 1.2). A small study of quenda in the south-west of WA found 0% (n=3) seropositive for antibodies to *T. gondii* using IHA (Jakob-Hoff and Dunsmore 1983). A more recent WA study around the Perth region found an average seroprevalence in quenda of 3.2% (2.9-3.7%) (n=158) using MAT (Hillman *et al.* 2017b). A recent study found 0% (n=6) seroprevalence in southern brown bandicoots on Kangaroo Island, South Australia (SA) (Taggart 2019). This is the only reported southern brown bandicoot seroprevalence study. There have been no seroprevalence studies performed in Victorian southern brown bandicoots.

In Darwin, Northern Territory (NT), 22.2% (n=12) of trapped northern brown bandicoots, in apparently normal clinical health, were positive for antibodies to *T. gondii* using MAT (Reiss *et al.* 2015). A 1992 Victorian study of eastern barred bandicoots in Victorian Zoos and Gellibrand Hill Park (both wild caught and

captive born) found a prevalence of 9% (n=57) using MAT, with a further 33% grouped as suspect, using the DAT (Miller *et al.* 2000). In the largest, longitudinal, seroprevalence survey of any bandicoot species (n=133), Obendorf *et al.* (1996) found an overall seroprevalence of 6.7% in Tasmanian eastern barred bandicoots (with positive results using both DAT and MAT). They also found that those bandicoots with demonstrable antibodies were less likely to be re-trapped and concluded that “eastern barred bandicoots were likely to die from primary *T. gondii* infection”, although there may be other reasons to account for the inability to re-trap (Obendorf *et al.* 1996).

Necropsy findings from the experimental infection of eastern barred bandicoots demonstrated gross pathology and histopathology consistent to that described in other mammals (Bettioli *et al.* 2000a). Lesions were present in a number of organs and tissues, including lungs, gastro-intestinal tract, lymph nodes, liver, spleen, pancreas, cardiac, skeletal and smooth muscles with histological evidence of areas of interstitial inflammation, focal necrosis in association with the presence of tachyzoites and bradyzoite tissue cysts (Bettioli *et al.* 2000a). Of note, the oocysts used in these studies were sourced from a virulent strain in USA (P89/VEG strain) and may not be representative of the genotypes found in Australia nor a typical naturally acquired dose. Similar necropsy findings in naturally infected eastern barred bandicoots are reported by others, with typical lesions most common in the brain, heart and skeletal muscle (Booth *et al.* 1995; Lenghaus *et al.* 1990a)(Samuel, J, 2018. Pers comm.). In a necropsy series on opportunistically collected dead eastern barred bandicoots performed in the 1980s in the Hamilton region, south-west Victoria, *T. gondii* was identified as a potential population-threatening parasite (Lenghaus *et al.* 1990b). In a later series of 39 eastern barred bandicoots found dead in the same region, eleven (28.2%) of the animals had *T. gondii* cysts within their tissues, as identified by histological examination, and active toxoplasmosis was diagnosed as being the primary cause of death in two of these cases (Samuel, J. 2018. Pers comm.). A wider necropsy series of eastern barred bandicoots sourced from Zoos Victoria, Taronga Zoo, private practices and the Hamilton Department of Agriculture

found toxoplasmosis to be the most prevalent infectious disease and the primary cause of death in 4 out of 136 (2.9%) bandicoots (Booth *et al.* 1995). In two other necropsy cases infection with *T. gondii* was identified incidentally (Booth *et al.* 1995).

In a necropsy series of 29 southern brown bandicoots, found between 2011 and 2014 from the Victorian Koo Wee Rup district, 15 had tissues tested for *T. gondii* by nested PCR (Jansen 2017). One of the 15 (6.6%) individuals tested, which died as the result of motor vehicle impact, was found to have a positive brain sample but had no discernible gross pathological or histological lesions. This represents the first documented case of a southern brown bandicoot recording a positive PCR for *T. gondii* DNA. Tissues from all 29 bandicoots underwent histopathological examination with none showing evidence of infection. Apart from isolated necropsy reports in southern brown bandicoots (Lynch 2010) this is the only reported necropsy series in southern brown bandicoots.

It must be noted that necropsy studies cannot give accurate indications of the prevalence, morbidity or mortality from infection with *T. gondii* in free-ranging populations, with results only representing those animals examined. Many animals will recover from infection while others may die without being found (Wobeser 2007). Recovery of animals that have died in the wild is notoriously difficult as most animals retreat to secluded places when they are sick, hence are not in visual sight of passers-by. Equally, many sick or dead animals will either be preyed upon or scavenged and hence remain undetected (Peisley *et al.* 2017; Wobeser 2007). However, a combination of gross necropsy, histopathology and molecular methods can identify animals that have acute, chronic or latent toxoplasmosis. In many cases diagnosis is only made by histopathology, which, as discussed earlier, has lower sensitivity and specificity compared with molecular methods (Wyrosdick and Schaefer 2015). Necropsy can also inform about other causes of death; co-morbid disease and parasitic infections.

Toxoplasma gondii is the most prevalent of the disease agents with potentially fatal outcomes that is reported in bandicoot species, (Bettioli *et al.* 2000a; Booth *et al.* 1995; Groenewegen *et al.* 2017; Hillman *et al.* 2017b; Lenghaus *et al.* 1990b; Obendorf and Munday 1990; Obendorf *et al.* 1996) (Samuel, J, 2018. Pers comm). Therefore, knowledge on the level of environmental oocyst contamination and likely exposure of potentially affected species would provide important information for management, such as feral cat management programs, Disease Risk Analyses (DRA) and translocation programs. However, there are currently no validated, practical approaches to evaluate this.

1.4 Measuring environmental contamination with *Toxoplasma gondii*

A variety of direct and indirect methods have been used to measure environmental contamination with *T. gondii* oocysts. Direct methods include microscopic soil oocyst counts and molecular analysis of the prevalence of *T. gondii* oocysts in soil, while indirect methods include feline prevalence of infection and density studies as well as the use of sentinel species, including native wildlife and pest species.

Direct methods

Microscopic oocyst soil counts

The most basic method for measuring soil contamination is to directly count the number of *T. gondii* oocyst in the soil, using standard faecal flotation methods and light microscopy. However, this is notoriously difficult and has a threshold of detection of 1,000 oocysts/gram of faeces (Dubey 2010; Lélou *et al.* 2011). Distortions occur when flotation solutions above 1.18 specific gravity are used and it is possible to misidentify *T. gondii* oocysts with *Hammondia hammondi* or *Neospora caninum* oocysts (Chemoh *et al.* 2016; Dubey 2010).

Molecular (qPCR) assessment of soil

A more specific and sensitive method of measurement of soil contamination has been to use molecular methods. The technique, described by Lélou *et al.* (2011), recovers oocysts from soil samples using a graduated diffusion technique, followed by DNA extraction and PCR to identify the presence *T. gondii* DNA. This method, repeated in other studies (Gotteland *et al.* 2014; Lélou *et al.* 2012), and recently refined (Escotte-Binet *et al.* 2019), is capable of detecting as few as 10-100 *T. gondii* oocysts/g soil (Lélou *et al.* 2011). Sporulation stage and age affects the efficiency of recovery of the oocysts from the soil with younger, sporulated oocysts more easily recovered (Lélou *et al.* 2012). Over time there is an increased loss of oocyst viability and detectability (using both microscope and qPCR methods) in dry soils compared with moist soils, while those soils with less than 40% sand appear to have a better recovery rate of oocysts (Lélou *et al.* 2012). Mineral composition has also been shown to affect the recovery of *T. gondii* oocysts and it is recommended that this should be taken into account when measuring different sites (Lélou *et al.* 2011).

Indirect methods

Feline prevalence of infection and density studies:

Knowledge of cat density and prevalence of infection with *T. gondii*, are valuable when estimating the contamination loads of *T. gondii* in the environment (Simon *et al.* 2016). Environmental contamination has been estimated using a range of scenarios of feline faecal sampling. The first, examined faecal samples of cats in an area, to determine the percentage containing oocysts and the concentration of oocysts per gram of faeces (Dabritz *et al.* 2007). The tonnage of faecal material contaminating the environment was then estimated using cat density estimates. A more conservative method assumed 0.9% cats were shedding oocysts at any point in time and that a conservative figure of one million oocysts were shed / infection (Dabritz *et al.* 2007). Using cat density values, it was estimated that 94 to 4,671 oocysts/m² were added annually to the environment and that 3 to 145 billion oocysts were being washed into Morro Bay, California, per year, hence

becoming a source of infection for the southern Californian sea otter (*Enhydra lutris nereis*) (Dabritz *et al.* 2007). Feline density has also been used to estimate *T. gondii* oocyst burden per square metre. This is achieved by combining cat density studies and seroprevalence studies and multiplying the number of domestic cat seroconversions / square kilometre /year by 1 million and 50 million to establish a range, assuming that infected cats shed between 1 and 50 million oocysts per year (Afonso *et al.* 2010). However, Gotteland (2014), found that there was not necessarily a direct correlation between oocyst distribution and cat density observations, with cat faeces and oocysts being found in areas where visual observations of cats had not occurred.

Prevalence of feline infection with *T. gondii* in Australia is correlated with topography and environmental conditions such as temperature, rainfall and humidity, with a higher prevalence occurring in cooler, wetter areas (Fancourt and Jackson 2014; Jakob-Hoff and Dunsmore 1983). A recent, Australia-wide, study tested feral cats for the presence of *T. gondii* DNA using qPCR on selected tissues. A positive correlation was identified between prevalence of infection with lower mean annual temperature and higher annual precipitation, but not with cat density (Dickson 2018). The highest prevalence records in Australian cats are from islands, particularly those with cooler, wetter climates: Christmas Island (96%) (Adams *et al.* 2008); Kangaroo Island, SA (89.4%) (O'Callaghan *et al.* 2005); Phillip Island, Victoria (90.2%) (Adriaanse 2018) and Tasmania (84.2%) (Fancourt *et al.* 2014; Hollings *et al.* 2013). This high prevalence may also reflect higher densities of cats on islands (Legge *et al.* 2017; Taggart *et al.* 2019). In contrast, in warmer and drier south-west WA, 32.4% of cats on average were found to have a positive serum titre (Jakob-Hoff and Dunsmore 1983), with prevalence higher in adult cats and higher in areas with higher rainfall. Similarly, an earlier Victorian study found a seroprevalence of 43.8% in the central district and 13.6% in the drier and hotter northern Mallee and Riverina plains (Coman *et al.* 1981). There has been no difference of prevalence of infection with *T. gondii* found in cats with an urban, domestic lifestyle compared to a more rural, hunting lifestyle (Jakob-Hoff and Dunsmore 1983). A Melbourne-based study also found

no difference between prevalence in cats living within 30km of Melbourne and those living further than 30km from the centre of Melbourne, with an overall seroprevalence of 39% (n=103) (Sumner and Ackland 1999). Dickson (2018), likewise generally did not find a correlation between prevalence of infection with *T. gondii* with urbanisation, the exception being urban areas in NT where increases were associated with cat density during the wet season.

There have been some Australian studies which have incorporated cat density and prevalence of infection in native marsupials with *T. gondii* to predict environmental contamination loads of *T. gondii*. Tasmanian studies on eastern quolls (Fancourt and Jackson 2014; Fancourt *et al.* 2014), and pademelons and spotted-tailed quolls (Hollings *et al.* 2013) found a positive correlation between cat density and seroprevalence. Similar seroprevalence studies of possums in New South Wales (Eymann *et al.* 2006) and northern brown bandicoots in NT (Reiss *et al.* 2015) concluded that cat density increases the risk of exposure of wildlife to *T. gondii*.

Sentinel species:

Sentinel species have been used to provide estimates of environmental contamination with pathogens (Nugent 2014; Psaroulaki 2010). A sentinel species is a species that informs about environmental contamination with a particular toxin or disease agent. In the context of assessing environmental burdens of *T. gondii*, an ideal sentinel would be:

- susceptible to infection by *T. gondii* but unlikely to become clinically ill or to die; giving a more accurate assessment of prevalence of infection
- living in a similar ecological niche to the target species; hence more likely to reflect the environmental contamination that the target species is exposed to.
- likely to have the same risk of ingesting *T. gondii* oocysts as the target species; hence more likely to accurately reflect the prevalence of infection in the target species.

- numerous; in order to maximise sample sizes to give a more accurate prevalence assessment.
- short-lived; providing information relating to more recent timeframes
- small; smaller animals typically have shorter lives and are easier to capture, handle, sample and store
- small territorial range; providing more accurate information about a particular geographic area
- readily and easily sampled; simplifying the sampling process and increasing the likelihood of larger sample numbers
- non-indigenous; if a lethal manner of assessing prevalence is used, or if capture and handling is likely to be more time and cost consuming or adversely affect individuals

Seroprevalence of antibodies to *T. gondii* in wildlife has been used to identify the presence of *T. gondii* in the environment, particularly when the parasite does not cause high morbidity or mortality in the sentinel species, such as in higher order predators (Fancourt *et al.* 2014). For these reasons, several authors have suggested that raptors (Love *et al.* 2016), black bears (*Ursus americanus*) (Dubey *et al.* 2015), arctic foxes (*Alopex lagopus*) (Prestrud *et al.* 2007) and red foxes (Verin *et al.* 2013) could serve as possible sentinel species. However, these species do not fit all the criteria for sentinel species mentioned above and the logistics involved in sampling carnivorous, high order predators are significant and costly, including human safety measures. It can be difficult to source large enough numbers to form statistically relevant conclusions (Richter *et al.* 2018), particularly when the environment is difficult to traverse and the target species is rare, threatened or endangered. In addition, seroprevalence studies will not necessarily reflect recent environmental conditions as higher order predators tend to bio-accumulate *T. gondii* and have the opportunity to develop antibodies over a long time-frame (Afonso *et al.* 2007), due to their relatively long life spans (in Australia: up to eight years for dingoes (Jackson 2003b); up to five years for the red fox (Anonymous 2017a); averaging 15.8 years for domestic cats (Teng *et al.* 2018) and up to 20 years for wedge-tailed eagles (Anonymous 2017b)).

Prey species, such as white-tailed deer (*Odocoileus virginianus*), have also been used to identify contaminated environments and potential zoonoses (Ballash *et al.* 2015). However, large herbivores, such as deer, are not ideal sentinel species, as they are long lived, have large territorial ranges and may not inhabit the same ecological niches and have the same foraging behaviour as the target species. In addition, large herbivores represent practical difficulties of immobilising large animals to sample and of obtaining significant numbers for statistical purposes.

Miller *et al.* (2000) suggest that evidence of *T. gondii* infection in bandicoot species can act as an indicator for “poor environmental quality”. Bi-valve shellfish, such as green-lipped mussels (*Perna canaliculus*) in New Zealand waters, fulfil most of the criteria for an ideal sentinel species and have been identified as potential bio-indicator sentinels for the presence of *T. gondii* in environmental waterways (Coupe *et al.* 2018).

Whilst the above-mentioned species have been mooted as potential environmental indicators for *T. gondii*, there have been no specific studies to identify and investigate an ideal indicator species. In the Australian setting, introduced, feral, pest species such as the house mouse and the European rabbit (*Oryctolagus cuniculus*), fit the requirements listed above of an ideal indicator species for *T. gondii* exposure in bandicoots.

Mice

House mice are understood to have arrived in Australia with European contact, possibly as early as 1606 when the Dutch first landed at Cape York (Reisman and Canfield 2017). They are now ubiquitous across the continent and able to live in a range of environments (Holland and Bennett 2007). Numbers fluctuate wildly, often to plague proportions, particularly following high food availability with good grain harvests (Singleton *et al.* 2001). This is attributed to their ability to alter litter size, increase length of breeding season and to begin breeding at

younger ages (Singleton *et al.* 2001). House mice are considered a serious pest species and can destroy grain and fodder crops, as well as invade sheds, storage facilities and houses. Arguably, it was the rapid spread and acclimatisation of the house mouse which contributed to the proliferation of cat ownership early in the European settlement of Australia, resulting in the enormous feral cat problem we have today (Abbott 2008). Mice have evolved as one of the most common intermediate hosts for *T. gondii*, presumably ingesting sporulated oocysts as they forage through soil and ground litter, although it is not fully understood how they become infected in the wild (Dubey 2010). Mice are a potential sentinel species for the presence of *T. gondii* oocysts in bandicoot habitats as typically free-ranging mice do not develop clinical toxoplasmosis and die, but mount an antibody response after infection (Dubey 2010). They inhabit similar microenvironments to bandicoot species (Coates, T. 2019. Pers comm.) and have some overlap in foraging behaviour (Bomford 1987; Tann *et al.* 1991). In addition, they are a non-indigenous pest species, small, short-lived, easily captured, large in number and have small home ranges (Mikesic and Drickamer 1992).

Worldwide, rodent studies have found prevalence of infection with *T. gondii* ranging from 1% to 59% (Brillhart *et al.* 1994; Buddhirongawatr *et al.* 2016; Hurkova-Hofmannova *et al.* 2014; Machacova *et al.* 2016; Normaznah *et al.* 2015). Rats have been postulated as an indicator species for *T. gondii* in Cyprus (Psaroulaki *et al.* 2010). Some laboratory strains of mice can maintain infection with *T. gondii* through vertical transmission for many generations without external re-infection with oocysts and without developing antibodies, however, few studies have examined this in the wild and none in Australia (Dubey 2010). Vertical transmission was demonstrated in mice living in English dwellings, where 59% (n=200) of mouse brains were found to have *T. gondii* by PCR studies but only one had mounted an antibody response (and hence been exposed to an oral route of infection) (Marshall *et al.* 2004). Prevalence of infection with *T. gondii* increases the closer a rodent is trapped to a dwelling as well as with age, which suggests there is at least some degree of post-natal

infection (Dabritz *et al.* 2008). However, it has been shown that mice, due to their smaller body size and shorter life-span, are less likely to be infected with *T. gondii* than rabbits (Afonso *et al.* 2007).

There have only been two Australian studies of prevalence of infection with *T. gondii* in free-ranging mice. An early, small (n=4), survey of mice from inland Queensland reported 0% seroprevalence (Smith and Munday 1965). A second, recent study found 0% seroprevalence in free ranging house mice (n=55) and black rats (*Rattus rattus*) (n=10), sourced from both the mainland and Kangaroo Island, SA (Taggart 2019). This study also found 0% (n=169) prevalence in bush rats (*Rattus fuscipes*) on the mainland but 2% (n=46) on Kangaroo Island.

While mice fit the criteria for an ideal sentinel species to reflect Australian environmental contamination with *T. gondii* oocysts, there have been no reported studies to specifically explore this and no studies demonstrating infection in Australian mice. There are no published studies that correlate prevalence of infection with *T. gondii* in rats or mice with the prevalence of *T. gondii* oocysts in soil.

Rabbits

As is the case with mice, introduced rabbits fulfil the criteria listed above to be a potential sentinel species for the presence of environmental contamination with *T. gondii*. First introduced to mainland Australia at Werribee, Victoria in 1859 by Thomas Austin for the purpose of hunting (Abbott 2011), the rabbit quickly acclimatised and spread throughout the country and is now found in all areas, across a range of habitats, other than the most northern area (Australian Government 2011). The rapid spread of the feral cat through Australia was facilitated by the ensuing rabbit plagues (Abbott 2008). Rabbits are considered a feral pest by wildlife managers and farmers alike, as they have the capacity of reaching plague proportions and compete with stock and wildlife for herbaceous

food resources and damage native plants (Australian Government 2011). The range of rabbits overlaps with that of most small, ground dwelling marsupials (Australian Government 2011), such as the southern brown bandicoot (Coates *et al.* 2008). Their grazing habit of cropping grass close to the ground and eating roots and seeds in the soil (Australian Government 2011) should, at least theoretically, increase the chance of ingesting infective *T. gondii* oocysts (Cox 1981). As they are relatively short lived and have a very high fecundity (Anonymous 2008; Peacock and Sinclair 2009), prevalence of infection with *T. gondii* should reflect recent environmental loads of oocysts. Additionally, their small home range enables a local identification of *T. gondii* environmental contamination (Cox 1981). Rabbits are capable of being infected with *T. gondii* (Dubey 2010) with the prevalence of antibodies to *T. gondii* in free-ranging rabbits in Europe varying from 6.1% to 53.8% (Dubey 2010). Rabbits have been shown to be susceptible to clinical toxoplasmosis following experimental oral infection with oocysts (Dubey 2010) but apart from a few isolated reports in individual animals in the USA (Dubey *et al.* 1992; Lehman *et al.* 1992), and epizootic outbreaks in Scandinavian hares (*Lepus europaeus* and *Lepus timidus*) (Gustafsson and Uggla 1994; Jokelainen *et al.* 2011), there have been no reports of clinical epidemics of toxoplasmosis in free-ranging European rabbits in other countries (Dubey 2010). We can therefore expect that free-ranging European rabbits in Australia are unlikely to die as a result of infection with *T. gondii*, and the measured prevalence of *T. gondii* in a population will reflect a true prevalence of infection and reflect the level of environmental contamination.

There have been very few rabbit *T. gondii* prevalence studies conducted in Australia. A 1972 Tasmanian serological study found that 17.4% of rabbits tested positive for *T. gondii* and the author suggested that rabbits may act as a reservoir of infection (Munday 1972). In south-west WA, a seroprevalence of 0% (n=57) was found over two distinctly different climatic areas using the IHA test (Jakob-Hoff and Dunsmore 1983). The most extensive Australian seroprevalence study, using IFT in free-ranging rabbits, was conducted in Victoria between 1971 and

1980 (n=1697), representing a range of geographic locations (Cox 1981). Five regions had rabbits with high titres of antibody (above 1:1000), ranging from 0.9% to 26%. There was a high correlation between seroprevalence and cooler and wetter locations, along with proximity to human population (Cox 1981).

A more recent, unpublished study (Harris 2011), measured seroprevalence in rabbits (n=145) in three locations: Yookamurra Sanctuary, 130km NE of Adelaide, SA; the Flinders Ranges, SA; and the Grampians, Victoria. Using LAT and ELISA methods, the study compared areas with two presumed different cat densities at each location. This study found that seroprevalence was higher in rabbits sourced from areas with presumed higher cat density and hence presumed environmental *T. gondii* contamination. It concludes that rabbits may be good measures of environmental contamination with *T. gondii* oocysts, even in hot, arid conditions. However, as sample numbers at each location were not high and exact cat densities were not performed, these findings are indicative only and should be replicated. A recent study conducted on Phillip Island, Victoria, found a prevalence of 10.5% (n=134) using qPCR of tissue samples of rabbits obtained from rabbit control programs (Adriaanse 2018). Adriaanse (2018) further demonstrated that rabbits may be a potential sentinel species for environmental contamination with *T. gondii* by comparing these results with prevalence of infection with *T. gondii* in free-ranging cats and prevalence of *T. gondii* oocysts in the soil.

As with mice, further work is required to establish whether rabbits can act as reliable indicator species for environmental contamination with *T. gondii* and whether prevalence of infection can reflect differing levels of soil oocyst burdens. If this proves to be the case, then rabbits could be a very useful, easily accessed species which can help inform conservation management decisions, including DRA for translocations of endangered small marsupials, such as the southern brown bandicoot.

1.5 Conclusion

In summary, Australia has a history of increased mammalian extinction and decline since European settlement, with a complex interaction of threatening processes leading to this loss (McKenzie *et al.* 2007; Woinarski *et al.* 2015). Disease agents can be either a major or contributing factor to species decline (Scheele *et al.* 2019). *Toxoplasma gondii* is an intra-cellular parasite, spread in the environment in the faeces of its' definitive host, the cat, and can infect warm-blooded vertebrate species world-wide, including Australian mammals, which act as intermediate hosts (Dubey 2010). Some species appear to be more susceptible to developing clinical toxoplasmosis following infection, compared to others (Jokelainen *et al.* 2011). Infection can result in a range of clinical symptoms including subclinical infection; mild illness; acute illness with severe pulmonary, gastro-intestinal and neurological symptoms and death (Dubey 2010).

The southern brown bandicoot is a small ground dwelling marsupial with a contracting range and is listed as endangered under the EPBC Act (Coates *et al.* 2008). There is only scant knowledge of health and disease agents associated with the southern brown bandicoot, however, *T. gondii* has been identified as a systemic parasite which can cause illness and death in other Australian bandicoot species (Booth *et al.* 1995; Lynch 2010; Obendorf and Munday 1990), although some animals are able to mount an immune response and survive (Hillman *et al.* 2017b; Obendorf *et al.* 1996). While there are a few studies of seroprevalence of antibodies to *T. gondii* in bandicoot species (Hillman *et al.* 2017b; Obendorf *et al.* 1996), there is only one current study in the southern brown bandicoot which was conducted in SA with a low sample number (n=6) (Taggart 2019). There are no Victorian southern brown bandicoot seroprevalence studies and only one necropsy series. There remain gaps in our knowledge for all bandicoot species as to the prevalence of initial infection, immune response, morbidity and mortality. There also remain gaps in understanding the effects endemic infection with *T. gondii* may have on population health, demographics and fecundity.

Conservation management of small, endangered, marsupial species such as the southern brown bandicoot, requires a good understanding of potential disease threats, including environmental contamination with *T. gondii*. The use of indicator species, which could inform about environmental contamination with *T. gondii*, such as mice and rabbits, would be a valuable tool for conservation management of endangered and threatened small mammals.

Given the knowledge gaps, future work should investigate the causes of mortality in southern brown bandicoots, with a particular emphasis on the role of toxoplasmosis and investigate seroprevalence of antibodies to *T. gondii* in free ranging populations. In addition, methods should be developed to allow assessment of environmental contamination with *T. gondii*, including the use of soil analysis and sentinel species.

1.6 Aims of this Thesis

The overall aim of this thesis is to investigate the role of *T. gondii* in the health of the endangered southern brown bandicoot in free-ranging populations in the northern hinterland of Western Port, Victoria and to develop conservation tools to assess the probability of infection of small marsupials with *T. gondii*.

Chapter Two aims to understand the causes of death in southern brown bandicoots, through an opportunistic necropsy series, with a focus on identifying evidence of infection with *T. gondii*.

Chapter Three aims to investigate further evidence of infection with *T. gondii* in two distinct southern brown bandicoot populations, through a seroprevalence survey of antibodies to *T. gondii*. It also aims to investigate the probability of infection with *T. gondii* in these bandicoot populations by employing eutherian species as sentinels and measuring environmental soil *T. gondii* oocyst burdens using molecular methods.

Chapter Four discusses the conclusions from these studies, summarising the achievements and knowledge gained, reviewing the limitations and making recommendations for future research.

CHAPTER TWO

MORTALITY INVESTIGATIONS IN SOUTHERN BROWN BANDICOOTS (*ISOODON OBESULUS OBESULUS*)

2.1 Introduction:

Many Australian wildlife populations have experienced decreasing abundance, range and diversity since European settlement, with 27 mammalian species having become extinct (Woinarski *et al.* 2015). The Threatened Species Scientific Committee lists a further ten mammals as critically endangered, 37 as endangered and 60 as vulnerable under the Environment Protection, Biodiversity and Conservation (EPBC) Act 1999 (Threatened Species Scientific Committee 2016a). A range of inter-related threatening processes that are currently impacting Australian mammal populations were discussed in Chapter One (Abbott 2008; Abbott 2011; Burbidge *et al.* 2008; McKenzie *et al.* 2007; Woinarski *et al.* 2015). Disease has been identified as a key threatening process in some Australian fauna, most notably, chytridiomycosis, caused by the fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, which has resulted in the extinction and decline in population size and distribution of multiple amphibian species (Berger *et al.* 2005; Scheele *et al.* 2019; Skerratt *et al.* 2010). Likewise, Devil Facial Tumour Disease (DFTD) is recognized as being responsible for dramatic population decline in Tasmanian devils (*Sarcophilus harrisii*) (Pye *et al.* 2016a).

Necropsies are an essential tool in population health surveillance and mortality outbreak investigations (Munson and Karesh 2002). Not only can necropsies ascertain the cause of death, they can also document reproductive and nutritional status, parasite species, the presence and indicative prevalence of various pathogens and other concurrent disease processes. They consequently make a significant contribution to the knowledge base of an endangered species and their disease processes and can make recommendations for future

management strategies (Ladds 2009; Speight *et al.* 2018; Vogelnest and Woods 2008).

Southern brown bandicoot (Isoodon obesulus obesulus)

The endangered southern brown bandicoot, a small omnivorous, ground-dwelling marsupial, persists in small free-ranging populations on the northern shores, islands and hinterland of Western Port (70km south-east of Melbourne) (Bryant *et al.* 2018; Coates *et al.* 2008; Maclagan *et al.* 2018) (Figure 2.1).

Whilst there have been sporadic necropsy reports of the southern brown bandicoot, most of our knowledge on potential diseases of significance is inferred from quenda (*Isoodon obesulus fusciventer*); the eastern barred bandicoot (*Perameles gunnii*); the western barred bandicoot (*Perameles bougainville*); and the northern brown bandicoot (*Isoodon macrourus*) (Booth and McCracken 1995; Lynch 2010). Diseases that have been identified as potentially important in southern brown bandicoots are discussed in Chapter One and presented in Table 1.1. The most common known causes of death in Victorian southern brown bandicoots are motor vehicle trauma, predation and ejection of pouch young when stressed (Coates, 2018, Pers. comm.) (Jackson 2003a; Jansen 2017; Robley *et al.* 2014). However, other likely causes include infectious disease, such as *Toxoplasma gondii*, which has been shown to cause illness and death in eastern barred bandicoots (Bettioli *et al.* 2000a; Groenewegen *et al.* 2017; Obendorf and Munday 1990) and is present in the southern Victorian landscape (Cox 1981).

Toxoplasma gondii

A wide range of species have been reported to be infected with *T. gondii* worldwide, with infection in non-Australian species mostly appearing to be asymptomatic or mild, although some species are particularly susceptible (Dubey 2010; Jokelainen *et al.* 2011). Australian mammals are known to

experience a range of clinical signs following infection, with many reported cases of illness and severe disease resulting in death of individual animals (Canfield *et al.* 1990; Donahoe *et al.* 2015; McOrist and Smales 1986). However, apart from Fancourt (2014), there has been little research undertaken to determine whether *T. gondii* is a significant factor driving Australian fauna population declines (Hillman *et al.* 2016).

Toxoplasma gondii is one of the most significant identified pathogens for eastern barred bandicoots, causing illness in experimental (Bettioli *et al.* 2000a), free-ranging (Groenewegen *et al.* 2017; Lenghaus *et al.* 1990b; Obendorf *et al.* 1996) and captive situations (Booth and McCracken 1995). Infected animals show symptoms ranging from subclinical disease to severe illness and even sudden death, with ocular, neurological and gastrointestinal symptoms (Bettioli *et al.* 2000a; Obendorf *et al.* 1996). It is unknown whether southern brown bandicoots are similarly affected and if infection with *T. gondii* is responsible for population declines in this species.

The most sensitive and specific post-mortem diagnosis for the presence of *T. gondii* is by tissue digestion and DNA extraction followed by quantitative (real time) polymerase chain reaction (qPCR) on selected tissues, in particular brain, eye, tongue, skeletal muscle, cardiac muscle and lung (Dubey 2010) (provided that the tissues selected contain the organism). Gross necropsy and examination of histopathological sections is also used to diagnose *T. gondii* infection, although it is less sensitive and specific. Sensitivity is enhanced with immunohistochemistry staining using polyclonal antibodies (Dubey 2010). Acute toxoplasmosis is diagnosed by the presence of crescent shaped tachyzoites, measuring 2 x 6 µm within host cells with an associated inflammatory response (Dubey 2010). In chronic or latent infection, *T. gondii* bradyzoites are identified within intracellular cysts, varying from 5µm in diameter, containing two bradyzoites to 100µm containing thousands of bradyzoites (Dubey 2010). The specific detailed size and shape of the intra-cellular parasite, as described by

Dubey (2010), differentiates between other possible apicomplexan infections including *Cystoisospora felis*, *Neospora canis* and *Besnoitia* spp. While bradyzoites, such as those of *C. felis* (also excreted in feline faeces), potentially could be present in marsupial tissues, there is no record of them in Australian wildlife. *Cystoisospora felis* sporozoites do not replicate in paratenic host cells and if found are a single parasite in a thick cyst wall typically in the mesenteric lymph node (Dubey 2018).

There are significant knowledge gaps in the role of toxoplasmosis in bandicoot population health and demographics, including prevalence and severity of infection and whether it is likely to pose a significant conservation threat for remaining populations (Hillman *et al.* 2016).

Study aims

The paucity of disease information for southern brown bandicoots represents a significant knowledge gap in this species. The aim of the present study was to investigate and contribute to the understanding of the causes of death and disease in free-ranging southern brown bandicoot populations. To achieve this, opportunistic necropsies were performed, including determination of cause of death where possible, identification of detected parasites, and qPCR testing for the presence of *T. gondii* DNA.

2.2 Materials and Methods

Study animals

Over a five-year period, from 29/10/12 to 26/1/18, twenty-six dead southern brown bandicoots were opportunistically collected by staff from inside the Royal Botanic Gardens Cranbourne (RBGC) (-38.127307, 145.285873) where they were found dead on roadways, except for one, which was found dead in a waterhole (W1122-17). The RBGC has a predator-proof ex-closure fence and no

cats have been detected there for several years (Nicholls, 2018)(T. Coates, 2018 Pers. comm.). A further five animals were found dead on roads close to the RBGC: one on Ballarto Road West, Cranbourne; and four on the Cranbourne-Frankston Road, Cranbourne. One bandicoot (W501-17) was recovered from Tynong (-38.087012, 145.625836), 74 km south east of Melbourne, next to a native nursery and another (W 1117-17) from a private property in Bayles (-38.187885, 145.584183), 78 km south-east of Melbourne (see Figure 2.1). While some animals were collected within hours of death, others had been dead for several days prior to collection. Some had been scavenged post-mortem. Free-ranging cats are known to be present across the landscapes where the remaining bandicoots were sourced (Nicholls, 2018) (S. Maclagan, 2018. Pers. comm.).

Collection date, location and any known clinical history were recorded. The bandicoots were transported either chilled on ice or frozen to the Faculty of Veterinary and Agricultural Science, University of Melbourne, where they were stored at -20°C. In some instances, animals were thawed and frozen twice.

Gross necropsy

Bandicoots were thawed for necropsy, after which they were weighed and sex and age were determined. Animals were classified as pinkies (unfurred, pouch young), un-weaned juvenile (weight < 160g), sub-adult (weight < 400 g) and adult (Duffy and Rose 2008; Jackson 2003a). Body mass measurements did not necessarily reflect live values, as in many cases cadavers appeared desiccated, and many were incomplete as a result of scavenging, in which case age allocations were made according to general physical appearance, including size and development (Duffy and Rose 2008). Body mass is thus only reported for complete bandicoot carcasses. Body condition was scored out of 5 using a basic method, similar to that described by Hillman et al (2017) (Table 2.1 and Figure 2.2). In some cases when the necropsy was performed by another person or when the carcass was too damaged to make an accurate determination, body condition was not recorded.

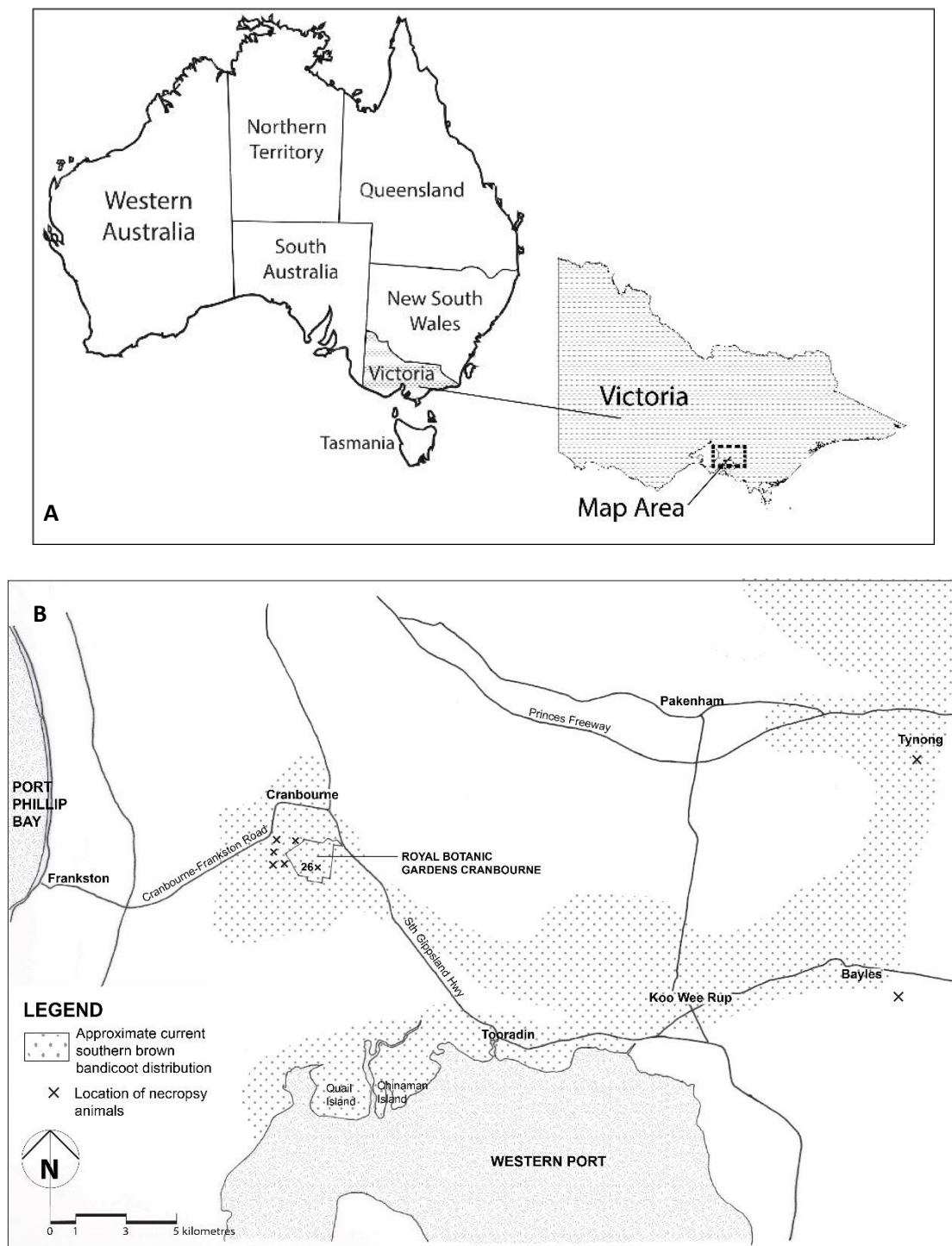


Figure 2.1 Remnant population sites for wild southern brown bandicoots (*Isoodon obesulus obesulus*) on northern Western Port, Victoria, Australia (A) and approximate current distribution with locations where southern brown bandicoots were found for the necropsy series presented here (indicated by x) (B)

Table 2.1 Body Score description for southern brown bandicoot (*Isoodon obesulus* *obesulus*) necropsy, modified from Hillman (2017)

Body Score	Description
1	Emaciated –no subcutaneous fat; virtually no musculature present; skeletal structures excessively prominent
2	Poor - no subcutaneous fat present; very poor musculature present; lumbar spine, scapulae and pelvis protruding and easily palpable
3	Good – very little subcutaneous fat present; well-defined musculature; skeletal structures palpable but not excessively so
4	Excellent – subcutaneous fat present; excellent, well developed musculature present, especially along dorsal spine, between shoulder blades and over pelvic area; skeletal structures just palpable
5	Obese – rolls of subcutaneous fat; excellent musculature; skeletal framework barely palpable

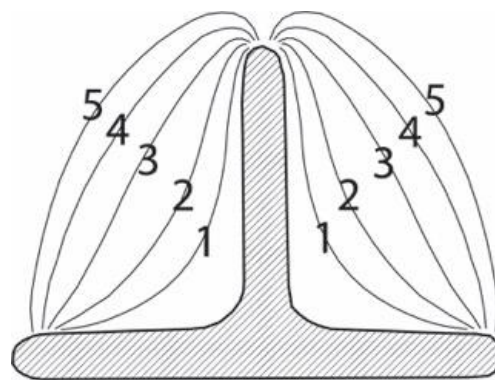


Figure 2.2 – Diagrammatic representation of vertebral cross-section showing musculature of body condition scores modified from Hillman (2017)

A full necropsy was performed, and gross lesions were systematically recorded. The condition of the integument, including lesions, contusions, haemorrhage, inflammation and alopecia were noted. The presence of fractures and any other traumatic damage was recorded. Any ectoparasites detected were removed and stored in 70% ethanol for later identification. The skin covering the thorax and abdomen was reflected dorsally to identify cutaneous and subcutaneous lesions, including possible penetration by predators or post-mortem scavenging. The gastro-intestinal tract (GIT) was removed en bloc (from the distal oesophagus to the rectum). It was longitudinally reflected, and contents were washed with tap water and allowed to settle. The supernatant was serially decanted and recovered helminths were stored in 70% ethanol for later identification. Tissue samples of approximately one cm³ from brain, heart, liver, lung, kidney, tongue, skeletal muscle and GIT were sampled and stored in 10% buffered formalin. In many cases, cadavers were so severely damaged or autolysed that tissues were not sampled for histopathology.

Further tissue samples were and frozen at -20 °C for subsequent real-time qPCR. These included the remainder of the brain, a whole eye, the remainder of the tongue, a whole kidney, approximately one cm³ of lung, liver, heart and skeletal muscle (where tissues were available).

Histopathology

Formalin-preserved tissue samples were embedded in paraffin, sectioned and stained routinely with haematoxylin and eosin. With the help of a veterinary pathologist, they were examined for histopathological lesions and the presence of pathogens, including *T. gondii* bradyzoites and tachyzoites. Some samples were too autolyzed to allow for histopathological interpretation of their sections. One set of tissues later had a Gram stain performed as many bacteria were identified in lung and diaphragm sections.

Ectoparasites

Fleas and mites were mounted on microscope slides and cleared with lactophenol for at least 48 hours before identification was attempted. They were examined under an Olympus BH2 microscope (Tokyo, Japan) and identified to species level using taxonomic references (Domrow 1987; Dunnet and Mardon 1974). Ticks were examined using a Nikon SM2 745 (Tokyo, Japan) dissecting microscope and identified to species level, where possible, using morphological features and taxonomic keys (Laan *et al.* 2011; Roberts 1969; Roberts 1970).

Endoparasites were identified with the assistance of a veterinary parasitologist and taxonomic keys (Spratt and Beveridge 2016) based on morphological features, using gross specimens examined under an Olympus BH2 microscope (Tokyo, Japan) and the histological sections stained with haematoxylin and eosin.

Tissue digestion and DNA extraction

In order to extract DNA from the southern brown bandicoot tissues, 5 g of pooled tissue from the brain, eye, tongue, heart muscle, skeletal muscle, kidney, liver and lung was added to 10 ml of Tris - EDTA lysis buffer (40 mM Tris, 10m MEDTA – Sigma Aldrich, St Louis, Missouri, USA), vortexed and incubated for 10 minutes in a 90 °C water bath. Once allowed to cool to room temperature, 200 µl of Proteinase K 20 mg/ml (Promega Corporation, Madison, Wisconsin, USA) was added and the sample re-vortexed. The sample was incubated overnight in a water bath at 55°C and subsequently completely homogenized using a mechanical homogeniser (T10 Basic Tissue Homogeniser, IKA-Werke, Staufen im Breisgau, Germany), after which 300 µl of the homogenized crude lysate was added to an equal amount of AL buffer (Qiagen Buffer AL, Qiagen, Hilden, Germany) in a 1.5 ml microfuge tube. The mechanical homogeniser was thoroughly cleaned with 10 % bleach and then thoroughly rinsed with distilled water between samples. Samples were incubated for 10 minutes at 70 °C and

cellular debris pelleted by centrifugation at 14,000 rpm for one minute. 380 µl of supernatant was transferred to a clean 1.5 ml microfuge tube containing 200 µl of 96-100 % molecular biology grade ethanol and vortexed. Further steps were followed according to the Qiagen DNA Blood and Tissue DNA extraction kit (Qiagen, Hilden, Germany) instructions, except that only 200 µl of AE Buffer was used in the final elution step. Samples were incubated at room temperature for one minute prior to the final centrifugation step. DNA was stored in a 1.5 ml Eppendorf tube at -20°C for qPCR.

Real time polymerase chain reaction

DNA from southern brown bandicoot tissue was subject to a multiplex Taq-Man based real time PCR targeting *T. gondii*, mammalian DNA as an extraction control and Equine Herpes Virus 4 (EHV4) as an internal qPCR control. Primers, probes, their gene targets and final concentrations are outlined in Table 2.2.

The qPCR was run in 20µl volumes containing GoTaq® Probe qPCR Master mix (Promega, Madison, WI) with 1µl of known quantity of EHV4, and 2µl of template DNA. Equine Herpes Virus genomic DNA added to the master mix, was sourced from cultured cells (provided by Dr Carol Hartley, Faculty Veterinary and Agricultural Science, The University of Melbourne). *T. gondii* genomic DNA sourced from culture (provided by Dr Chris Tonkin, Walter Eliza Hall Institute), along with mammalian probes and primers, were used as positive controls for each qPCR run. All primers and probes were sourced from Integrated DNA Technologies (IDT, Coralville, Iowa, USA). A MIC Personal qPCR Cyclor (Bio Molecular Systems, Upper Coomera, Queensland, Australia) was used to perform the reactions. The first cycle was set for incubation at 95 °C for two minutes, and there followed 40 cycles of alternating denaturation at 95 °C for 15 seconds and annealing/ extension at 60 °C for 60 seconds. Cycling analysis was set to 'dynamic', with a Cq threshold of 0.01 (Automatic) and a fluorescence cut-off level of 5%, except for EHV which had a fluorescence cut-off of 20%. The first cycle was ignored.

Serial dilutions of known *T. gondii* positive samples were run using the full protocol and combined mammal synthetic DNA (G Block) of the mammalian 18S ribosomal RNA gene: 5' – GGC GGC GAC GAC CCA TTC GAA CGT CTG CCC TAT CAA CTT TCG ATG GTA GTC GCC GTG CCT ACC ATG GTG ACC ACG GGT GAC GGG GAA TCA GGG TTC GAT TCC GGA GAG GGA GCC TGA GAA ACG GCT ACC ACA TCC AAG GAA GGC AGC AG – 3' (Integrated DNA Technologies) and *T. gondii* DNA. A Cq 36.11 was identified, at which concentrations of 0.00005 pg/μl were the lowest dilution that a sample reached fluorescence detection threshold and was reliably identified as positive. A sample was deemed to be positive if both wells were positive. If only one well was recorded as positive, the sample was repeated, and the sample was subsequently considered negative if both wells were negative. A qPCR was deemed a failure if the EHV in the sample was greater by two or more cycles compared with the negative control EHV Ct value.

Table 2.2 Protocol for qPCR for *Toxoplasma gondii* DNA using mammalian tissues

	Sequence	Gene target	Concentration per reaction	Citation
Toxorep Probe	5Cy5/ ACG CTT TCC /TAO/ TCG TGG TGA TGG CG/ 31AbRQSp/		150nM	
Toxorep forward primer	5' – AGA GAC ACC GGA ATG CGA TCT -3'	529 bp highly repetitive element	150nM	(Lélu <i>et al.</i> 2012)
Toxorep reverse primer	CCC TCT TCT CCA CTC TTC AAT TCT		150nM	
EHV Probe	5'- /5HEX/ TTT CGC GTG /ZEN/ CCT CCT CCA G/ 31ABkFQ/ -3'		100nM	
EHV Forward Primer	5'- GAT GAC ACT AGC GAC TTC GA -3'	gB	40nM	(Ng-Nguyen <i>et al.</i> 2017)
EHV Reverse Primer	5'- CAG GGC AGA AAC CAT AGA CA -3'		40nM	
Mammalian Probe	5'- /56-FAM/ CGT GCC TAC /ZEN / CAT GGT GAC CAC /31ABkFQ/ -3'		350nM	
Mammalian Forward primer	5'- CGA ACG TCT GCC CTA TCA AC -3'	18S ribosomal RNA	250nM	This study
Mammalian Reverse Primer	5'- CGT TTC TCA GGC TCC CTC T - 3'		250nM	

2.3 Results

Study animals

Necropsies were performed on 33 southern brown bandicoots, with Figure 2.1B indicating where they were found. Fifteen of the bandicoots were female, including one juvenile and one sub-adult; four adults had evidence of recent active reproduction, with enlarged pouch and enlarged, distended teats. Thirteen bandicoots were male, including a juvenile and three sub-adults. Three animals were so damaged that their sex was indeterminate, but one was probably a sub-adult and the remaining two were probably adults. Five animals were still at the early, furless pouch young stage, of which three were males, while the sex of the other two was not determined. Male adult body mass ranged from 820.5 g to 1,302.0 g with an average of 788.3 g and a median of 747.2 g (n = 9). Female adult mass ranged from 407.5 g to 674.0 g with an average of 521.9g and a median of 604.5g (n=13). Unfortunately, body condition and mass were not measured in all cases, as mentioned in Methods. But for the remaining animals, body condition scores ranged from 1 to 5 (including juveniles and sub-adults but excluding unfurred pouch young), with a median score of 3 (n = 19). Sex, weight and body scores are detailed in Appendix 2.1.

Gross necropsy

The overwhelming majority of southern brown bandicoots showed injuries consistent with trauma. These are detailed in Appendix 2.1. The gross necropsy findings of the 22 cases which were found on roads all had a range of traumatic injuries including multiple fractures, especially of skull, mandibles, ribs and pelvis. In most cases (n = 17) there was thoracic haemorrhage and some destruction of lung parenchyma and displacement and gross damage to the heart consistent with crush injuries and fractured ribs. Nine cases had a ruptured diaphragm, with herniation of the abdominal organs into the thoracic cavity. In four cases the abdominal wall was also ruptured with evisceration of the GIT. The stomach was ruptured with spillage of contents in nine cases. In six instances the abdominal organs were un-identifiable, obliterated or else were

missing, presumably due to post-mortem scavenging. Post-mortem scavenging was differentiated from traumatic injuries by the presence of puncture wounds, matting of hair, tearing lesions of tissues and lack of haemorrhage associated with traumatic lesions.

The remaining three trauma cases had lesions consistent with predation, including puncture wounds in the ventral cervical area, around the head and over the dorsal thoracic area; soft tissue damage of ventral cervical musculature and intercostal muscles; and fractures of the cervical vertebrae, skull and mandibles. These lesions are consistent with those described in other predated marsupials (Ladds 2009). Post-mortem scavenging was differentiated from predation injuries by the lack of associated ante-mortem haemorrhage or contusions.

Several individual cases were of interest. Bandicoot W 1117-17, recovered from Bayles with a history of sudden death, had approximately one ml of blood flowing from the nose and mouth. There was no apparent evidence of predator-related puncture wounds. The chest cavity contained more than 25 ml of free bloody fluid and the lungs were very dark, congested and collapsed. The abdominal cavity contained about 3 ml of free blood.

The left uterus of W 410-16 was found to be quite enlarged (4cm x 1.5 cm) with thick, pale, creamy contents consistent with a pyometra. There were no smears taken or gram stains done, but samples were taken for histopathology. The head of the animal was missing along with muscles from the cervical vertebrae and the thoracic contents. This was thought to be due to post-mortem scavenging by wildlife. Case W 1120-17 was found to have a very distended stomach full of food grains, mostly lentils, dried peas and soy grits with no other abnormalities detected. W 689-16 and W 1121-17 were two groups of unfurred pouch young which were very thin but had no other abnormalities detected.

A large number of helminths were present in the small intestine of W 1006-16, the small intestinal mucosa was inflamed, and the lumen of the small intestine was filled with a blood tinged fluid. The lungs were congested, and the stomach and intestines were unusually empty of ingesta, suggesting that the animal may not have eaten for some time. Several fleas were removed from its coat.

In three cases, W 1007-16, W 1028-16 and W 100-17, gross necropsy and tissue sampling for histopathology and qPCR were unable to be performed due to the severely damaged and decomposed nature of the carcasses. However, it is likely that they were victims of motor vehicle trauma, based on the fact that they were found beside roadways and the extremely damaged, flattened appearance of the bodies.

Histopathology

Significant histopathology findings are included in Appendix 2.1. In many cases, there was severe autolysis or freezing / thawing damage making reading and interpretation of slides difficult. Histopathology was unable to confirm pyometra in bandicoot W 410-16, because all tissues were severely autolysed. The uterine section showed no inflammation in the mucosa, but there was some debris on the mucosal surface and the oviduct showed occasional, scattered neutrophils, but no bacteria. Unfortunately, the bandicoot suspected of drowning (W 1122-17) was so autolysed that only muscle was submitted for histology and even this was too autolysed to assess. Histopathology was also not able to elucidate a cause of death for the bandicoot with dried lentils in its stomach and suspected bloating (W 1120-17). Histopathological examination of tissues from W 1117-17, which had large amounts of free blood in its chest, revealed an extensive, fibrinous pleuro-pneumonia along with active infection of the mediastinum and epicardium and non-suppurative serous inflammation of the diaphragm.

Gram staining of these tissues revealed the presence of bacteria, mostly gram-negative rods, associated with the pleura, cardiac membranes and diaphragm. This, together with the inflammation, was consistent with ante-mortem

infection. The source of this infection was most likely a penetrating injury, such as a cat bite, through the oesophagus or intestines, which then spread into the thoracic cavity. The copious free blood in the thorax may have been as a result of damage to a large vessel through the bite itself or through infection following the bite.

Histopathology was not able to add anything to the cases of suspected motor vehicle trauma. However, it was possible to identify several endoparasites and other incidental findings, such as mild prostatitis.

Table 2.3 Causes of mortality in a necropsy series of southern brown bandicoots (*Isoodon obesulus obesulus*) opportunistically collected dead from within the grounds of the Royal Botanic Gardens Cranbourne (Inside RBGC), the area immediately outside the RBGC, Tynong and Bayles (all Outside RBGC).

	MVT	Predation	Pouch ejection	Lentil ingestion	Pyometra	Drowning	Other
Inside RBGC	16	3	2	1	1	1	1
Outside RBGC	6	1					
Total	22	4	2	1	1	1	1

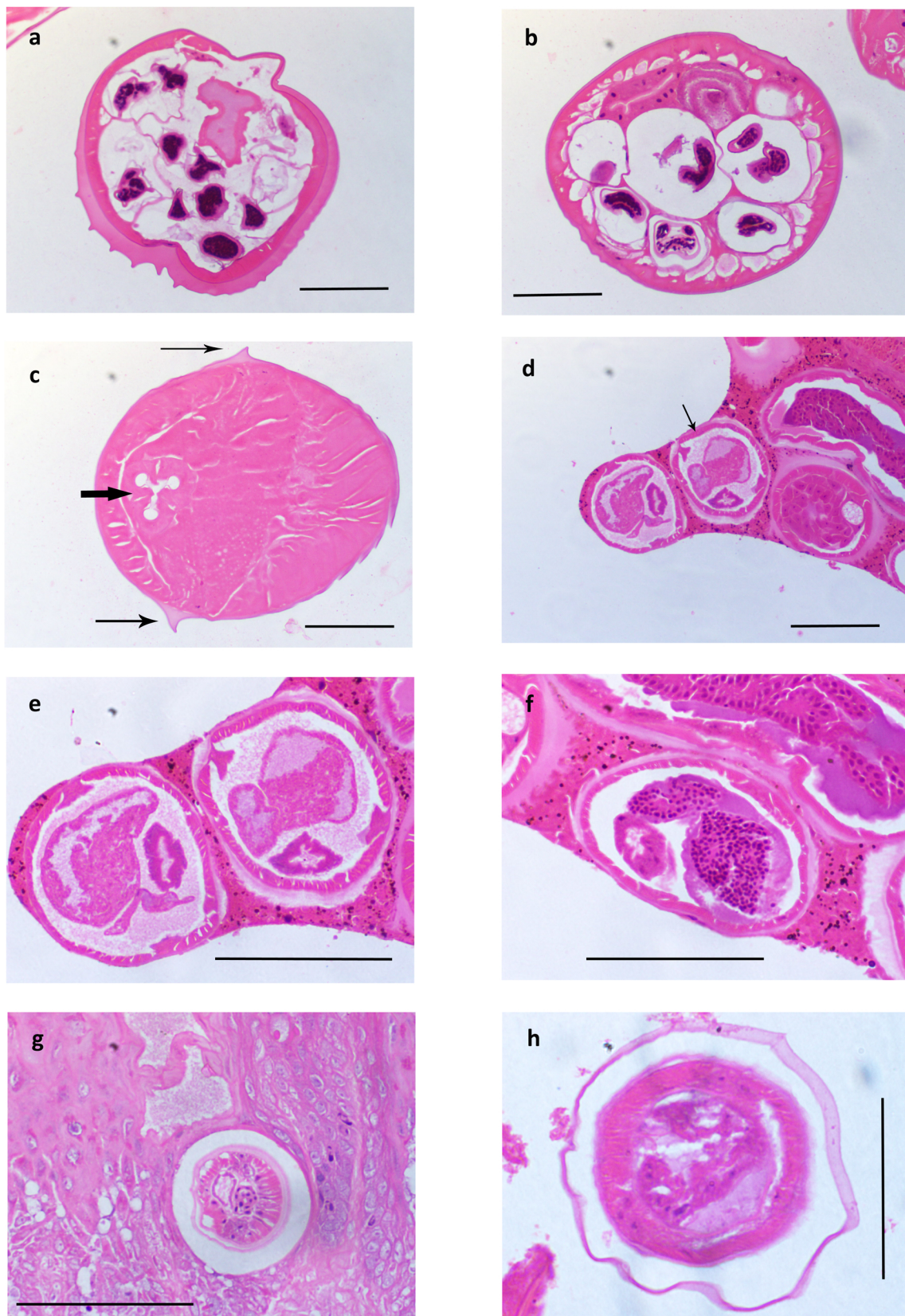


Plate 2.1. Light micrographs of helminths from opportunistically necropsied southern brown bandicoots (*Isodon obesulus obesulus*) from southern Victoria. a) - c) *Labiolura inglisi* in large intestine of W 411-16 (10x magnification); thin arrows indicate alae and fat arrow indicates transverse section of oesophagus. d) - f) cross-sections of metastrongyloid lungworm(s) surrounded by necrotic lung in W 1006 - 16 (d) x10; e) and f) x 20), arrow indicates worm cuticle; g) cross-section of *Eucoleus longiductus* in tongue of W 517-17 (20x); h) cross-section of a trichostrongyloid nematode found on tongue surface of W 1008 - 16 (40x). Black bar represents 1mm in all micrographs other than h) where it represents 0.5mm.

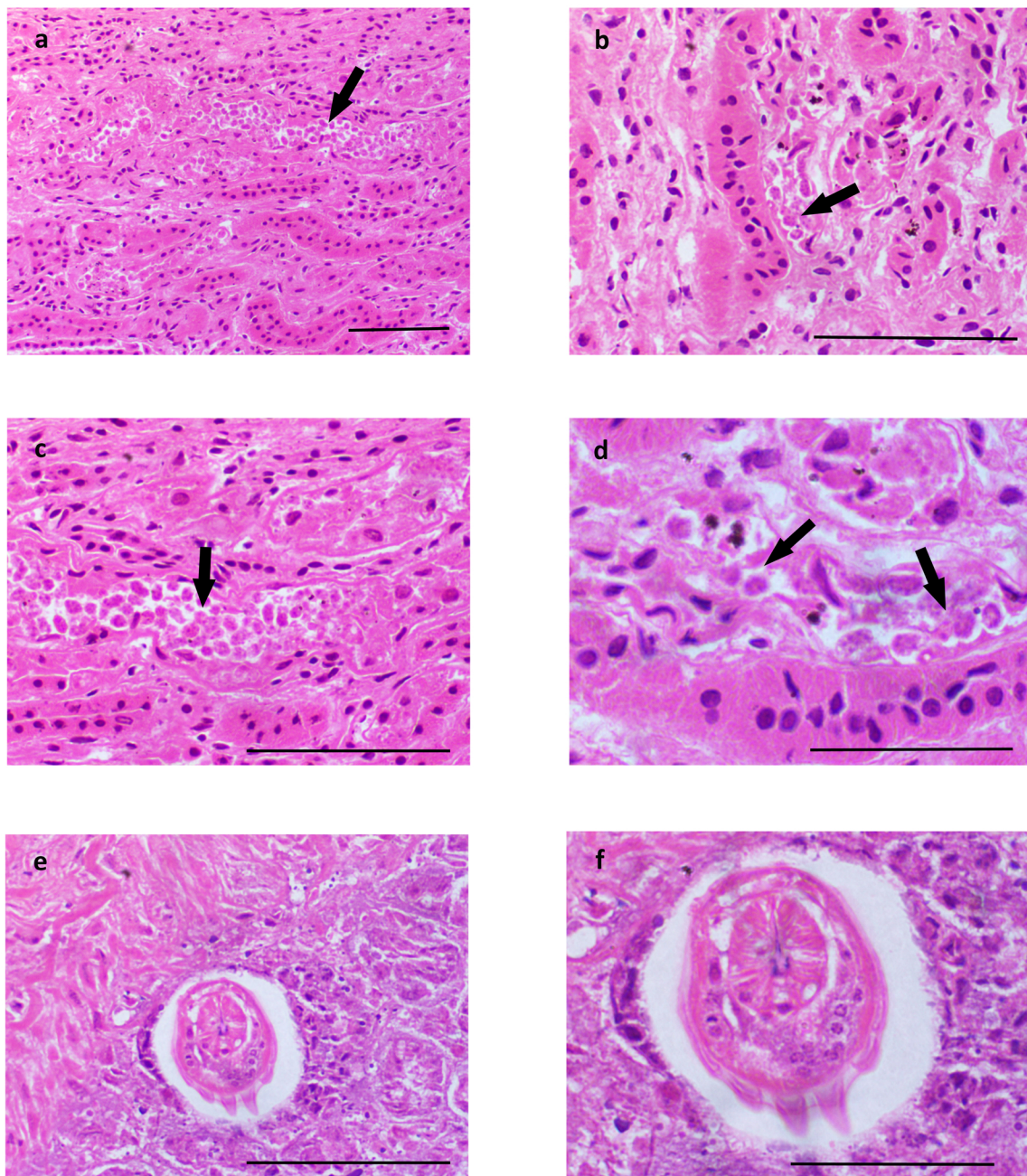


Plate 2.2. Light micrographs of internal parasites in opportunistically necropsied southern brown bandicoots (*Isodon obesulus obesulus*) from southern Victoria. *Klossiella quimrensis* in renal medullary tubules (arrows pointing to organisms): a) from W 1117 - 17 (x10 magnification); b) W 1117 - 17 (x20); c) from W 434 - 16 (x20); d) from W 434 - 16 (x40). *Mackerrastrongylus mawsonae* from W 517-17 embedded in glandular tissue of small intestine: e) note parasite is surrounded by an inflammatory reaction and necrotic tissue destruction, near junction between lamina muscularis (top left corner) and glands in submucosa (lower right corner) (x20); f) x 40. Black bars indicate 1mm in a) - e), and 0.5mm in f).

Endoparasites

Toxoplasma gondii

Tissue samples from 30 animals were tested for the presence of *T. gondii* DNA using real time quantitative PCR analysis. All samples were negative. Bandicoots W 690-16 and W669-16 displayed weak positive results (Cq 37) in one well only. When re-tested they were negative in both wells and hence were deemed to be negative. No animals showed evidence of infection with *T. gondii* on histological examination.

Other endoparasites

Several other endoparasites were identified by histopathology and low-powered microscopy in 15 bandicoots (see Table 2.2). In addition, un-identified nematode eggs were found on the gastric mucosal surface and within the lumens of the duodenum, small intestines and large intestines in four individuals. For one bandicoot, W 411-16, sections of nematodes in the duodenal lumen were identified as *Parastrongyloides australis*, and in the large intestine as *Labiolura inglisi* based on the features seen in histological examinations and identifications of entire parasites made from the same series of hosts (Plate 2.1 a, b, and c) (Spratt and Beveridge 2016). Several helminth sections were identified within the large bronchioles of the lung of W 1006-16, which are most likely a metastrongyloid species (Plate 2.1 d, e, and f). Bandicoot W 517-17 had nematodes embedded within the cornified epithelium of the tongue, identified as *Eucoleus longiductus* based on the features seen in histological examinations and identifications of entire parasites made from the same series of hosts (Spratt and Beveridge 2016) (Plate 2.1 g). In another bandicoot (W 1008-16), a nematode was detected above the papillae on the surface of the tongue. This is most likely a trichostrongylid nematode as it has a cuticle with a single longitudinal ridge (Plate 2.1 h) (Spratt and Beveridge 2016).

The coccidian *Klossiella quimrensis* (Plate 2.2 a, b, c, and d) was identified in the tubules of the renal medulla of five individuals. All of these were adult males, except for one adult female. They were all sourced from RBGC, except for an adult male (W 1117-17) from Bayles.

Embedded in the glandular tissue of the small intestine of W 517-17, (the same animal with *Eucoleus longiductus*), a species of *Mackerrastrongylus* was identified, which is most likely to be *M. mawsonae* (Plate 2.2 e and f), based on adults previously recovered from the small intestine lumen of other bandicoot species in southern Australian zones (Beveridge, I., 2019 Pers comm) (Spratt and Beveridge 2016). There was a surrounding inflammatory reaction and evidence of necrotic tissue destruction caused by the parasite. A nematode egg was found on the surface of the oesophagus of W 409-16.

Intestinal helminths were recovered from intestinal washings of several animals. Seven different species were identified from 11 individuals, all from the RBGC (Table 2.4). Three of these bandicoots were sub-adults. *Labiobulura inglisi* (Nematoda: Subuluroidea) was the most commonly represented, being present in seven of the animals, while *Linstowinema* sp (most likely *L. inglisi*) were found in a further three individuals. *Australiformis semoni* (Acanthocephala) and *Trichuris peramelis* (Nematoda: Trichinelloidea) were present in two individuals, while *Physaloptera* sp (Nematoda: Physalopteroidea) was identified in W 411-16. *Potorolepis peramelidarum* (Cestoda: Hymenolepididae) and *Peramelistrongylus skedatos* (Nematoda: Trichostrongyloidea) were each identified only once. Some southern brown bandicoots had co-infections with several endoparasite species, for detail see Table 2.4.

In many cases the GIT was either ruptured, macerated, severely autolysed, mummified or missing, so recovery of endoparasites was not possible.

Table 2.4 Endoparasites found in a necropsy series of southern brown bandicoots (*Isoodon obesulus obesulus*) from The Royal Botanic Gardens Cranbourne, Cranbourne, Tynong and Bayles.

	409-16	411-16	433-16	434-16	699-16	688-16	690-16	691-16	1006-16	1008-16	1009-16	517-17	1117-17	1120-17	185-18
Cestodes		•													
<i>Potorolepis peramelidarum</i>	•														
Acanthocephala															
Acanthocephalan sp.	•														
<i>Australiformis semoni</i>												•			
Nematodes															
<i>Linstowinema</i> sp					•			•							•
<i>Physaloptera</i> sp		•													
<i>Mackerris strongylus</i>												•			
<i>Peramelistrongylus skedatos</i>												•			
<i>Parastrongyloides australis</i>		•													
<i>Labiolura</i> sp			•		•	•	•								•
<i>Labiolura inglisi</i>		•							•	•	•				
<i>Trichuris peramelis</i>					•				•						
<i>Metastrongyloid</i> nematode in lungs									•						
Trichostrongyle (tongue)										•					
<i>Eucoleus longiductus</i> (tongue)												•			
Coccidia															
<i>Klossiella quimrensis</i>				•				•			•		•	•	

Ectoparasites

Ectoparasites were collected from twelve animals (see Table 2.5). At least two species of ticks were identified. Most were nymphs and one was an adult that was missing its capitulum. There is currently no reliable way to differentiate the nymphs of *Ixodes hirsti* and *I. trichosuri* morphologically, so they could not be identified to species level (Laan *et al.* 2011). W 1122-17 had a tick with an elongated body, characteristic of *Haemaphysalis* spp, most likely *H. humerosa* (Roberts 1970). Unfortunately, the mouth parts were missing so full identification was not possible.

Two species of mesostigmatid mites (*Haemolaelaps marsupialis* and *Mesolaelaps antipodanus*) were identified, as well as a small number of trombiculid mites, which could not be identified to species level due to specimen damage. It is likely that many more mites were present on the bandicoots, but as they are more difficult to detect, they were probably often not collected and thus no prevalence estimates were attempted. A section of an unspecified mite was identified on histopathology in the skin of W 688-16. Three species of fleas (*Pygiopysylla zethi*, *P. hoplia* and *Stephanocircus dasyuri*) were identified. One animal was infested with two species of flea and another with three different flea species.

Table 2.5 Ectoparasite and endoparasite species identified on necropsy of southern brown bandicoots (*Isodon obesulus obesulus*)

Ectoparasites	408-16	409-16	411-16	699-16	670-16	690-16	691-16	1006-16	1008-16	517-17	1120-17	1122-17
Fleas												
<i>Pygiopysylla zethi</i>			•			•		•				
<i>Pygiopysylla hoplia</i>			•					•		•		
<i>Stephanocircus dasyuri</i>			•						•			
Mites												
Trombiculidae					•							
<i>Haemolaelaps marsupialis</i>		•										
Ticks												
<i>Ixodes</i> spp.	•	•	•				•			•		
<i>Ixodes hirsti/ trichosuri</i>	•			•		•				•	•	
<i>Ixodes cornuatus/ holocyclus</i>			•									
* <i>Haemaphysalis</i> sp.												•

*probably *H. humerosa*

2.4 Discussion

The present study detected a high number of southern brown bandicoot deaths consistent with motor vehicle trauma, including inside a predator-free conservation area. On the other hand, neither *T. gondii* nor active toxoplasmosis were detected in any of the examined animals.

Sex ratio

An almost equal number of male and female bandicoots were represented in this necropsy series. The sex ratio (0.86 males/female) was higher than that obtained by Maclagan (2018) in free-ranging, live populations, at remnant sites, who recorded 0.44 males per/ female (Maclagan *et al.* 2018). A possible reason for this could be that Maclagan was trapping live animals, whereas we were collecting animals found dead, mostly due to motor vehicle trauma. Male bandicoots have been shown to roam more widely within territories (Maclagan *et al.* 2018) and hence may be more likely to cross roads and be involved in collisions with vehicles, as has been shown with macropod road kill (Coulson 1997).

Motor vehicle trauma

The main suspected cause of death in this series was motor vehicle trauma (22 of 33 cases). The risk of southern brown bandicoot deaths by motor vehicle strikes at RBGC is increased by the large number of daily vehicle visitors to the site, the relatively large number of bandicoots active within the RBGC and the fact that many of the southern brown bandicoots have become habituated to humans and are diurnally active as well as crepuscular (Coates, 2018 pers comm.). While recent surveys and research work has been performed on southern brown bandicoot populations both inside and outside of the RBGC (Maclagan 2016; Nicholls *et al.* 2018), actual population size is difficult to determine, as is the impact motor vehicle trauma may be having on these populations. In 2018, the bandicoot population inside RBGC was estimated to be 50-60, based on camera and spotlighting surveillance (Coates T, pers comm., 2018). This figure has decreased from 200-300 over the years 2010-2013 with decline attributed to drought conditions and macropod overgrazing impacting on habitat quality (Coates T, pers comm., 2019). It is possible that motor vehicle trauma may now also be contributing to population decline. Motor vehicle trauma has been shown to be a population limiting factor in other species, such as koalas (*Phascolarctos cinereus*) and bare-nosed wombats (*Vombatus ursinus*), resulting in a significant

impact on the viability and genetic pool of populations (Griffith *et al.* 2013; Roger *et al.* 2011). Motor vehicle trauma has also been a significant issue in re-introductions of the Tasmanian devil in Tasmania (Grueber *et al.* 2017) and eastern quolls (*Dasyurus viverrinus*) translocated to Booderee National Park, NSW (Dexter 2019). While population effects of road kill can be masked by immigration from connected populations (Roger *et al.* 2007), this may not be the case with the southern brown bandicoot in the south-central Victorian region, which has distinct, unconnected populations due to severe habitat destruction and fragmentation (Coates *et al.* 2008). It is important that silent declines, such as those caused by road kill, are monitored and mitigated against (Roger and Ramp 2009).

A range of mitigation strategies can be employed to reduce wildlife roadkill. Limiting driving speed to less than 80 km/hour from dusk to dawn has been shown to reduce the incidence of road kill (Hobday 2010). Signage on open roads can be used to remind drivers of this, although the efficacy of this is not known. Under-road tunnels can be used effectively to enhance wildlife survival at road interfaces but must be designed correctly with the target species in mind to be effective (Van der Ree *et al.* 2015). Such tunnels have recently been constructed in the RBGC to mitigate roadside deaths, along with signage, speed limits and speed humps (Coates T. Pers comm. 2018). In addition, Cardinia Shire recently incorporated an amendment to the Environmental Significance Overlay for the urban growth areas of the townships of Bunyip, Garfield and Tynong (Cardinia Shire 2019) to protect and enhance the habitat of the southern brown bandicoot and to mitigate against motor vehicle trauma. This includes habitat protection and restoration in new housing developments as well as the building of tunnels under roads. While all the above-mentioned actions, along with continued community engagement and education on road speed, may have some effect in mitigating roadkill it is difficult to quantify and while bandicoots continue to live close to roadways, they will always be at risk of motor vehicle trauma.

Predation

Four cases in this necropsy series were thought to be either directly or indirectly linked to predation, three of which were from the RBGC. Southern brown bandicoots fall in the critical weight range that makes them very susceptible to predation by both cats and foxes (Abbott 2011; McKenzie *et al.* 2007; Woinarski *et al.* 2015). Additionally, their ground nesting and ground foraging behaviour makes them even more susceptible, as was found in the failure to re-introduce the western barred bandicoot to the Heirisson Prong Peninsula in WA (Short 2016). Conversely, quenda has made an excellent recovery moving from a Near Threatened conservation status to that of Least Concern, largely due to fox control programs (Woinarski *et al.* 2014a). It was somewhat surprising to find several cases linked to predation at the RBGC, given there is a predator exclusion fence in place. However, the Garden's entrance gates are open during visiting hours and potential breaches of the fence enable the entry of cats and foxes (Coates T. Pers comm. 2018). RBGC is currently undertaking further fence repairs and predator control programs, which will help to decrease these incidents (Coates T. Pers comm. 2018).

One bandicoot (W 1117-17) was of particular interest, as a member of the public reported that this animal was one of several southern brown bandicoots that had been found dead in her yard over the previous few months. The resident owned several cats but was convinced they did not harm the bandicoots. Histopathology results led to a conclusion that this animal had most likely died from bacterial pleuritis secondary to a penetrating bite wound. Whilst a puncture wound through the skin was not identified, in many cases the only external sign of predation is a slight matting of the hair (Bender *et al.* 2019). This case highlights the difficulty of educating the public that domestic cats, which may appear benign, are in fact capable of killing or inflicting mortal injuries on small wildlife.

For all native wildlife in the critical weight range (CWR) residing outside predator exclusion fences, predation by foxes, dogs and cats will always be a

significant population threat and control of these predators will remain an utmost priority (Woinarski *et al.* 2015), along with education of pet owners. In the Koo Wee Rup swamp area, the proximity of southern brown bandicoots to households adds to the complexity of their survival (Maclagan 2016). Encouragingly, it has been shown in Sydney that people interacting closely with bandicoots are more likely to engage in conservation behaviour (Dowle and Deane 2009). Education and engagement activities with communities living in association with free-ranging southern brown bandicoot populations may be a key factor in improving control of domestic cats and dogs and increasing feral animal control.

Other causes of death

No definitive cause of death was determined for the remaining animals, but two litters of unfurred pouch young were deemed to have died secondary to ejection from the pouch. Bandicoots are known to eject their young when distressed (Department of Environment and Conservation 2009). One bandicoot was diagnosed with pyometra on gross pathology, but this could not be confirmed as microbiology was not performed (due to the length of time between death and necropsy) and histopathology was inconclusive due to advanced autolysis of tissues. There was no other cause of death elucidated and it is unknown if the pyometra was an incidental finding or a primary pathological process leading to death. This is the first time that pyometra has been described in a southern brown bandicoot.

Another bandicoot was in very poor condition and found in a water hole, but because advanced autolysis precluded histopathological examination, it was difficult to confirm death by misadventure and drowning. This remains a likely scenario, however, possibly secondary to weakness from poor body condition. The final case (W 1120-17) was unusual in that the stomach was very bloated and distended containing a recent feed of material resembling dried lentils, split peas and soy-grit. It is possible that this may have caused the death either

through some sort of toxic reaction to these unusual food items or through gastric distension and bloating.

Toxoplasma gondii

Molecular methods did not detect *T. gondii* in any of the 30 necropsied southern brown bandicoots tested in this study, nor were any tissue cysts consistent with infection with this parasite detected on histopathology. Therefore, infection with *T. gondii* was eliminated as either a cause of death or a contributing factor to death in all cases.

Molecular methods of detection of *T. gondii* are considered the most reliable method of post mortem diagnosis (Dubey 2010). The qPCR used in the present study is highly sensitive and specific. The protocol we developed had a sensitivity of detecting *T. gondii* to a concentration of 0.000005 pg at a Cq of 36. Others have reported that PCR and qPCR can provide a detection limit of the equivalent of 0.01 tachyzoite / μ l (Suviriyapaisa *et al.* 2017). There were two individuals which recorded a positive in a single well with Cq of 37. When these two samples were each repeated, they were both negative in both wells and hence the result was recorded as a negative. However, some laboratories have recorded such results as a positive (Gotteland *et al.* 2014; L  lu *et al.* 2011; L  lu *et al.* 2012). There remains a small chance that there was a minute amount of DNA in the positive wells, however, this most likely occurred through contamination of the well during the laboratory procedure.

There is a chance that infected tissues were not selected for analysis, thus resulting in false negatives. The possibility of detecting *T. gondii* DNA was maximised by using 5 g of a combined range of tissues including central nervous tissue, skeletal muscle, heart, eye, lung, kidney and liver, which are the most likely tissues to contain *T. gondii* organisms (Costa *et al.* 2018; Di Cristina *et al.* 2008; Jur  nkov   *et al.* 2015), although in a few cases not all of these tissues were

available due to severe autolysis or scavenging. Based on all these considerations, it seems highly likely that the qPCR results presented here are true negatives.

As most animals in the present study were sourced from within the RBGC where no cats are present, it was not surprising that these animals tested negative for *T. gondii*. Those animals sourced from outside the RBGC, where there is a known, free-ranging feline presence, presumably had a higher chance of encountering soil contaminated with infective oocysts, nonetheless, no positives were detected. An unpublished report by Jansen (2017) tested tissues for *T. gondii* by PCR from 15 southern brown bandicoots from the same general locations as the present study and found one animal from Bayles to be positive. If these results were combined with those from the series reported here, then from January 2011 until February 2018, a total of 45 dead southern brown bandicoots from the Koo Wee Rup / Cranbourne areas were tested by either PCR or qPCR for the presence of *T. gondii* resulting in a prevalence of infection of 2.2%. This is roughly consistent with a recent study of trapped, live quenda in the Perth peri-urban and bushland areas, which found 3.7% (n=54) and 2.9% (n=104) respectively had IgG antibodies to *T. gondii* (Hillman *et al.* 2017b).

While this study did not find any evidence of infection with *T. gondii*, it is possible that infected southern brown bandicoots are dying as a result of acute toxoplasmosis or misadventure, including predation, related to the infection. They may then not be recovered for necropsy because they die remote from human observation or are subjected to post-mortem scavenging. Free-ranging eastern barred bandicoots both in Victoria and Tasmania have become infected with *T. gondii* and developed clinical toxoplasmosis, with some dying as a result (Samuel, J 2018, Pers comm) (Booth and McCracken 1995; Groenewegen *et al.* 2017; Obendorf *et al.* 1996). The eastern barred bandicoot has since become extinct in the wild on the mainland and it is possible that infection with *T. gondii* may have played a role in the final decline of the wild populations. This

underlines the importance of determining risk of exposure and prevalence of infection in remaining free-ranging populations of other bandicoot species if we are to prevent them from becoming extinct in the wild. More studies are required to ascertain if southern brown bandicoots are susceptible to clinical disease with *T. gondii* and to examine the resultant morbidity, mortality, pathology and immune responses. In addition, further cross-sectional and longitudinal studies are required to investigate the impact and prevalence of infection with *T. gondii* within free-ranging southern brown bandicoot populations. Further necropsy of opportunistically found dead bandicoots is recommended, including the use of PCR studies on tissues to estimate prevalence of infection, allowing for the fact that not all dead bandicoots will be recovered. These results can be compared with seroprevalence of IgG antibodies to *T. gondii* in live animals.

Further studies are recommended to develop methods of better assessing the risk of exposure to this disease. Given the difficulties associated with determining accurate infection levels in bandicoot populations themselves, this could be achieved through estimating environmental burdens of *T. gondii* oocysts, including free-ranging cat densities, directly measuring prevalence of oocysts in soil and measuring the prevalence of *T. gondii* infection in sentinel species in the landscape.

Endoparasites

A range of internal parasites was detected in the animals examined, with all except one previously reported and described in southern brown bandicoots. To the best of the author's knowledge, this is the first report of a lungworm in a southern brown bandicoot (Beveridge, I. Pers comm, 2018). Pulmonary nematodes have been reported in eastern barred bandicoots, but not identified (Spratt and Beveridge 2016). Two species of lungworm *Filostrongylus peramelis* and *Marsupostrongylus bronchialis* have been identified in the northern brown bandicoot (Spratt and Beveridge 2016). The *Physaloptera* sp. was identified to

genus level only, because only the female can be identified to species (Spratt and Beveridge 2016). The only other record of *Physaloptera* sp. in a southern brown bandicoot was not identified to species-level either (Spratt and Beveridge 2016). On the other hand, *Physaloptera peramelis* has been identified from northern brown bandicoots, and *P. thalacomys* from eastern barred bandicoots (Spratt and Beveridge 2016).

The coccidian parasite *Klossiella quimrensis* was identified in five bandicoots. This parasite has previously been identified in the renal tubules of bandicoots and was reported as occurring in one southern brown and three eastern barred bandicoots from Maria Island, Tasmania in 1975 (Barker *et al.* 1975). The only other documented report of *Klossiella* sp. in a southern brown bandicoot was associated with a recent necropsy series of southern brown bandicoots from the same geographic area as this study (Jansen 2017). *Klossiella quimrensis* was identified in 30% (n=20) of kidneys from a necropsy series of captive western barred bandicoots (Bennett *et al.* 2007). These infections were not associated with clinical disease. Similar incidental findings of *Klossiella* spp were noted in a necropsy series of eastern barred bandicoots from Hamilton from 1991-1994 (Samuel, J. Pers. comm. 2018). Whilst *K. quimrensis* is a common, incidental finding (Lynch 2010) (Samuel, J. Pers comm 2018), it is not often reported. As the pathogenesis of this species remains uncertain and, in the present series, the presence of *K. quimrensis* was not associated with other renal pathology, it should most likely be considered an incidental finding.

Ectoparasites

Most of the ectoparasites collected in the present study had previously been reported from bandicoots and are not considered to be significantly deleterious to the host (Beveridge and Spratt 1996; Hillman *et al.* 2017b; Spratt and Beveridge 2016). The tick species identified were considerably different to those identified by Hillman *et al.* (2017) on quenda in south-west West Australia, highlighting different host-parasite relationships based on geographical areas.

The long-bodied tick found was unfortunately missing mouth parts and was only able to be identified as *Haemophysalis* sp (Roberts 1970). However, it is most likely to be *H. humerosa*, which has not previously been reported from the east coast south of Sydney (Roberts 1970) (Beveridge, I. 2018. Pers. comm.). While opportunistic ectoparasite collection during necropsy is not necessarily representative of abundance and diversity on live hosts, it continues to expand our understanding of species distribution and prevalence, particularly in less extensively studied host species.

Limitations

The results presented here may not proportionally reflect the causes of all deaths in the sampled populations of southern barred bandicoot. Animals dying from other causes, such as old age or acute illness, may not be detected if they die under cover or if carcasses are removed by scavengers (Peisley *et al.* 2017). This necropsy series relied on staff at RBGC and members of the general public to report findings of dead animals. This may have biased the results towards those readily found near roadways and hence more likely to have been subject to motor vehicle trauma. Whilst staff at RBGC were aware and supportive of this project and readily supplied cadavers, there was some difficulty in raising awareness amongst the general public and there was very limited representation of animals from the wider free-ranging population. Similarly, many cases of predation would not be discovered as the prey is usually consumed by the predator (Wobeser 2007). Unless specific tracking techniques are used to follow individual animals, as was the case in an eastern barred bandicoot trial introduction at French Island (Groenewegen *et al.* 2017), it is impossible to know the fate of every animal. Nevertheless, opportunistic necropsies form an important contribution to wildlife health surveillance, and this particular series makes a valuable contribution to our understanding of the causes of death, health, parasites and the presence of different potential pathogens in the southern brown bandicoot.

The results were compromised by varying degrees of autolysis, as a result of cadavers often not being found until several days after death. This impacted on gross necropsy and histopathology results and endoparasite identification. Further compromise to histopathology results occurred through freezing and thawing of cadavers prior to examination. Likewise, many animals were so severely damaged that a full necropsy and histopathology could not be performed.

While oral, nasal and cloacal swabs were taken in most cases, these have, at the present time, not undergone viral or microbiological testing. However, such testing would be feasible in the future if indicated.

2.5 Conclusion

The present necropsy series found that 66.7% of animals died as a result of motor vehicle trauma and 12.1% as a result of predation. Further investment in public awareness campaigns in relation to road speeds, usage and road management mitigating actions, as well as domestic cat management (Elliott *et al.* 2019) and feral predator control would be useful strategies to attempt to reduce mortalities associated with these causes.

None of the cases examined were positive for *T. gondii* on qPCR or histological examination. As little is known of the effects of *T. gondii* infection in the southern brown bandicoot further studies are recommended with particular emphasis on the prevalence and consequences of infection with *T. gondii* at an individual and population level. In addition, research to establish a method of measuring environmental contamination with *T. gondii* in the Australian landscape is recommended. The identification of a suitable sentinel species for the presence of *T. gondii* in the environment would provide a valuable tool for managing free-ranging populations of southern brown bandicoots and other small marsupials in the Australian landscape.

Appendix 2.1 Gross necropsy and histopathology results for southern brown bandicoots (*Isoodon obesulus obesulus*) sourced from southern central Victoria.

Abbreviations: RBGC – Royal Botanic Gardens Cranbourne; F – female; M – male; R – right; L – left; DV – dorsoventral; # - fracture; NAD – no abnormalities detected; Sub cut – subcutaneous; Neg – negative; GIT – gastro-intestinal tract; SI – small intestine; LI – large intestine; DDx - differential diagnosis; ? – suspected but not confirmed cause of death.

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 408-16	17/12/2015	RBGC	juvenile	M	421		abdominal puncture wound extensive sub-cutaneous haemorrhage # R and L maxilla # cranium #R and L ribs ruptured stomach ruptured diaphragm	mild autolysis Tongue: areas of mild interstitial inflammation	Trauma - ? predation
W 409-16	17/04/2014	RBGC	sub-adult	M	402		empty stomach no fractures	Oesophagus: nematode egg on surface NAD	Unknown

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 410-16	17/04/2014	RBGC	adult	F	428.5 + (not complete)		head missing muscles missing from cervical vertebrae thorax contents missing L uterus: enlarged 4 x 1.5 cm, pale cream contents	Uterus: no inflammation on mucosa, some debris on surface Oviduct: occasional scattered neutrophils- no bacteria all tissues severely autolysed	? Pyometra
W 411-16	?2015	RBGC	adult	M	820.5		sub-cut haemorrhage ventral neck # skull and anterior cervical vertebrae mandible separated L eye enucleated gross damage to cervical vertebrae haemorrhage in thorax cestodes and nematodes in GIT	Gastric, SI and LI: several sections of nematodes and nematode eggs <i>Parastrongyloides australis</i> <i>Labiolura inglisi</i> . Lungs: traces of fluid on alveoli – agonal change	Trauma - ? predation

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 432-16	?	RBGC	adult	F	674		bilateral sub-cutaneous and superficial muscular haemorrhage over dorso-lateral thorax puncture wounds on L and R thorax tail missing # ribs damaged heart tear in diaphragm lung in abdomen intestines through abdominal hernia	SI and LI: helminths present urogenital tract: bands of bacteria and some inflammatory cells	Motor vehicle trauma and post-mortem scavenging

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 433-16	?	RBGC	adult	M	1023.5		abrasions ventral jaw and axilla R eye - white blood stained fluid in abdomen	Intestines: helminth in lumen <i>Labiolura</i> sp Lungs: moderately congested transudate fluid in alveoli Prostate: occasional very small foci of interstitial inflammation	Trauma – ? Motor vehicle
W 434-16	?	RBGC	adult	F-with 2 pouch young	604.5		# skull # L maxilla & mandible Lungs: mottled, red and congested Liver: diffuse and pale Intestines: ruptured	Lungs: congested with large number of macrophages in peripheral alveoli containing dust particles Intestines: nematodes in lumen Kidney: <i>Klossiella quimrensis</i> in renal tubules	Motor vehicle trauma

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 435-16	?	RBGC	adult	M	1302		Abdomen: sub - cutaneous haemorrhage # pelvis # L & R tibio-tarsus # lumbo-sacral-coccygeal vertebrae thorax- watery blood	Muscle: suspect <i>T. gondii</i> - like cyst but probably not Prostate: foci of non-suppurative inflammation	Motor vehicle trauma
W 699-16	2/12/2015	RBGC	adult	F	600	4	laterally flattened # skull # all L & R ribs ruptured diaphragm-intestines in chest ruptured abdominal wall lungs collapsed uterus and pouch and teats enlarged	NAD	Motor vehicle trauma

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 670-16	16/01/2015	RBGC	adult	F	450	2	# skull # mandibles # L ribs # pelvis R eye enucleated chest crushed	Lung: tissue damage and congestion tongue: simple trauma lesion	Motor vehicle trauma
W 688-16	30/12/2015	RBGC	sub-adult	F	300	2	# R ribs #L tibia # pelvis perforated abdominal wall herniated intestines ruptured diaphragm ruptured stomach chest crushed and destroyed	Skin: mite sections present in pit	Motor vehicle trauma
W 689-16	29/10/2012	RBGC	3 x pinkies	3 x M	20 20.5 20.5	0	some slight bruising around head and neck	nil	possible ejection from pouch

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 690 -16	17/08/2014	RBGC	adult	M	950	4	# skull # mandible # all L & R ribs diaphragmatic hernia GIT in chest cavity and contents ruptured other organs un-identifiable chest - contents crushed	Prostate: few scattered foci of non-suppurative interstitial inflammation.	Motor vehicle trauma

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 691-16	23/07/2015	RBGC	adult	M	1,150	2	2/3 tail missing # all L & R ribs L thoracic wall ruptured sub cut bruising over L chest wall and ventral abdomen Lungs: collapsed and crushed Diaphragm: ruptured Stomach: herniated into chest and ruptured	Kidneys: renal tubules contain <i>Klossiella quimrensis</i> Prostate: some areas of inflammation	Motor vehicle trauma
W 1006-16	2/04/2016	RBGC	sub-adult	M	529	3	no tail (old injury) lungs: congested Intestines: reddened mucosa, empty with blood tinged fluid many helminths in gut+ stomach: empty (unusual)	Lungs: congested, <i>Metastrongyloid</i> nematode in bronchiole Intestines: NAD	Unknown- ? Acute disease or poor nutrition and high parasite load

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 1007-16	28/01/2014	RBGC	adult	F	484	not measured	severe autolysis – impossible to perform necropsy # lumbar spine	Not done	Motor vehicle trauma
W 1008-16	3/04/2014	RBGC	adult	F	513	4	sub-cut haemorrhage # skull # mandibles # R ribs ruptured abdominal wall R eye enucleated ruptured diaphragm ruptured GIT	Autolysed NAD	Motor vehicle trauma
W 1009-16	4/03/2013	RBGC	adult	M	850.5	4	# skull enucleated R eye ruptured L abdominal wall lungs: congested GIT: ruptured	Autolysed Kidney: <i>Klossiella quimrensis</i> in renal tubules	Motor vehicle trauma

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 1028-16	?	RBGC	adult	?	?	?	Flattened, dehydrated and fossilised	Not done	Motor vehicle trauma
W 1029-16	?	RBGC	sub-adult	M	490.5	3	# skull # mandibles puncture wounds under throat very autolysed	Not done -too autolysed	Predation (cat)?
W 99-17	16/08/2016	outside RBGC Ballarto Road West	adult	?	311	?	#'s everywhere completely squashed and macerated	muscle only: NAD	Motor vehicle trauma
W 100-17	10/08/2016	outside RBGC corner Cranbourne -Frankston Rd and Pearcedale Road	? Sub-adult	?	185		macerated carcass unable to perform necropsy	Not done	Motor vehicle trauma

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 501-17	23/05/2017	Tynong- near garden nursery	adult	M	872.5	5	completely flattened laterally #mandible # skull	Lungs: congested	Motor vehicle trauma
W 517-17	12/05/2017	RBGC	adult	F	407.5	4	sub cut haemorrhage thorax and inguinal area # cervical vertebra # 3 x R ribs Tail: old de-gloved injury diaphragm ruptured intestines and liver herniated into chest cavity Intestines: reddened contents helminths inflamed intestinal mucosa	Tongue: nematodes <i>Eucoleus longiductus</i> SI: nematodes- <i>Mackerris strongylus</i>	Motor vehicle trauma

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 1117-17	26/11/2017	Bayles – Private rural house	adult	M	1255	5	lungs: dark, congested collapsed blood discharging from nose and mouth 25 ml free blood in chest cavity 3 ml free serosanguinous fluid in abdomen tongue: protruding, dark and tip was traumatised	heart: fibrinous epicarditis with mild to severe inflammatory reaction diaphragm: non-suppurative serous inflammation with mono-nuclear cells lung: fibrinous pleuro-pneumonia with pleuritis on both surfaces gram stain: thick layer of bacteria on pleura and diaphragm -mixed infection ante-mortem Kidneys: <i>Klossiella quimrensis</i> in renal tubules	Penetrating injury causing severe pleuropneumonia and infection in diaphragm and epicarditis Most likely a cat bite with secondary haemorrhage of large thoracic vessel DDx rodenticide

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 1118-17	6/10/2017	RBGC	adult	F	497.5	4	#'s throughout body, skull, vertebrae, ribs pelvis etc. DV flattened R eye enucleated thorax: completely crushed abdominal organs: missing	None	Motor vehicle trauma
W 1119-17	October '17	Outside Gardens Frankston-Cranbourne Rd	adult	F	644.5	3	# multiple vertebrae # all ribs # skull, mandibles bruising all muscles and soft tissue abdominal wall opened, contents missing	None Kidneys: <i>Klossiella quimrensis</i> in renal tubules	Motor vehicle trauma

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 1120-17	28/05/2017	RBGC	adult	M	1,091	5	R eye missing, L eye traumatised no fractures brain: liquified lungs: dark, congested, collapsed stomach: bloated, external haemorrhage and full of lentils, dried peas and soy grit Intestines: excessive gas	Kidneys: <i>Klossiella quimrensis</i> in renal tubules	? Bloat and toxicosis from lentil /pea/soy grit ingestion and secondary scavenging
W 1121-17	?	RBGC	2 x pinkies	?	4.5 12.5	0.5	very thin NAD	none	Ejection from pouch

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 1122-17	15/11/2017	Found in water RBGC	Juvenile	F	124.5	1	very thin no #s no soft tissue injuries muscles: pale Lungs: pale. Collapsed, autolysed hard to tell if water in alveoli	none	? Drowning found in water very poor condition
W 184-18	9/01/2018	outside gardens Pearcedale Road	adult	F	431	3	completely flattened #skull # tail # L humerus # pelvis dislocated L and R femoral heads chest: totally crushed abdomen: totally crushed and organs mashed	none	Motor vehicle trauma

Animal	Date Found	Location	Age	Sex	Body mass (g)	Body Score (1-5)	Gross Pathology	Histopathology	Cause of Death
W 185-18	25/01/2018	RBGC	adult	F	554.5	3	# skull eyes missing soft tissue bruising and trauma to ventral cervical area stomach: full of grey gritty material lungs: congested - 2ml free blood	NAD Liver: occasional small foci of inflammatory cells	Motor vehicle trauma
W 186-18	26/01/2018	outside RBGC Frankston-Cranbourne Rd	adult	F	496	3	# skull # L humerus # L femur # pelvis # tail and de-gloved chest: crushed and macerated abdomen: organs macerated stomach: ruptured	NAD	Motor vehicle trauma

CHAPTER THREE

ASSESSMENT OF ENVIRONMENTAL *TOXOPLASMA GONDII* BURDENS USING DIRECT SOIL MEASUREMENTS AND EUTHERIAN SENTINEL SPECIES AS INDICATORS.

A POTENTIAL CONSERVATION MANAGEMENT TOOL

3.1 Introduction

The southern brown bandicoot (*Isoodon obesulus obesulus*) is one of the many marsupials that has undergone significant decline in both range and population numbers since European colonisation. It is listed as 'endangered' under the federal Environment Protection and Biodiversity Conservation (EPBC) Act 1999 and 'threatened' under the Victorian Flora and Fauna Guarantee (VFFG) Act 1988 (Department of Environment Land Water & Planning 2016). Threatened populations of marsupials, such as the southern brown bandicoot, are the focus of significant conservation efforts (Woinarski *et al.* 2014a). Wildlife health is a key goal in management of these threatened populations and is dependent on a range of factors including habitat quality; food availability; population dynamics; genetic diversity; resilience to environmental change; predator pressure and disease (Hing *et al.* 2016b; Stephen 2014).

Disease risk

Disease has been identified as a cause of population decline in Australian fauna since the turn of the century, with the eastern quoll (*Dasyuris viverrinus*) being noted in 1922 to have dramatic die-offs as a result of disease, only surviving in isolated populations (Short and Calaby 2001). In the same year, koalas (*Phascolarctos cinereus*) were also noted to have had significant losses due to disease, although specific diagnoses were not recorded (Short and Calaby 2001).

Understanding disease risk is therefore an important consideration in wildlife management (IUCN 2013; Northover *et al.* 2019). Translocation and re-introduction

programs are commonly used conservation tools to manage wildlife populations. These are undertaken for two broad reasons: firstly, to restore populations by reinforcing existing populations with releases of the species into an existing population; or, secondly, to establish new populations through conservation translocations, either to areas where a species was formerly known to occur (a reintroduction) or to an area outside of its indigenous range (introduction) (IUCN 2013). A Disease Risk Analysis (DRA) should be undertaken prior to any such animal movements, according to the International Union for Conservation of Nature (IUCN) guidelines (IUCN 2013) and the 'Manual of Procedures for Wildlife Disease Risk Analysis' (Jakob-Hoff *et al.* 2014). A DRA is used to inform decision making (Hing *et al.* 2017) to avoid either a pathogen being introduced to a previously naïve population or a host being placed into an environment containing pathogens that may compromise that host (Rideout 2019). Disease Risk Analysis also plays a role in management decisions surrounding the minimisation of translocation stress, which may make an animal more susceptible to succumbing to disease (Vaughan-Higgins 2019).

The decisions made as part of a DRA are often based on limited knowledge about the prevalence and impacts of disease in wildlife populations, making accurate, specific assessments challenging (Vaughan-Higgins 2019). In particular, predicting the level of exposure to pathogens with indirect transmission pathways may be difficult, as this is likely to be affected by factors such as frequency and density of shedding, persistence under varying environmental conditions and probability of contact and infection (Lange *et al.* 2016). One such pathogen is the coccidian parasite *Toxoplasma gondii*, whose infective oocysts are shed into the environment by its definitive feline host (Dubey 2010). This parasite infects a wide range of intermediate hosts worldwide but appears to be particularly pathogenic in Australian marsupial species (Canfield *et al.* 1990; Donahoe *et al.* 2015; McOrist and Smales 1986).

Assessing exposure to Toxoplasma gondii

Southern brown bandicoots are among those that have been shown to be susceptible to infection with *T. gondii* (Hillman *et al.* 2017b; Jansen 2017). While clinical disease as a

result of infection is yet to be confirmed in this species, it is well documented in the eastern barred bandicoot (*Perameles gunnii*) (Bettioli *et al.* 2000a; Lenghaus *et al.* 1990b; Obendorf and Munday 1990), with neurological, optical and gastro-intestinal signs, ranging from mild disease to acute death (Bettioli *et al.* 2000a). Further, it has been suggested to have significant potential population impacts in that species (Lenghaus *et al.* 1990b). In the face of significant declines in both species, and with disease being a potential contributing factor, a clearer understanding of the level of environmental exposure of bandicoot populations to *T. gondii* is needed.

Necropsy and serological studies can provide valuable information about infection with *T. gondii*. Two necropsy series of south-central Victorian populations of southern brown bandicoots (Chapter Two) (Jansen 2017) revealed low levels of infection with *T. gondii*. However, these limited studies with their inherent sampling bias could not reliably inform about the actual prevalence in free-ranging live animals. Nor were they able to draw firm conclusions about the level of environmental contamination with *T. gondii* oocysts and hence probability of exposure.

Examination of live animals can provide further information. Morphometric measurements, for example, can indicate overall health of animals (Hayes and Shonkwiler 2001). Animals with clinical toxoplasmosis are likely to lose weight and have lower body mass and body condition scores (Groenewegen *et al.* 2017). These parameters, along with general clinical examination for other physical and behavioural symptoms (see Chapter One) provide further information about possible infection with *T. gondii* (Bettioli *et al.* 2000a; Obendorf and Munday 1990).

In addition, seroprevalence studies can further inform about prevalence of infection. There have been no seroprevalence studies investigating the presence of antibodies to *T. gondii* in Victorian southern brown bandicoots. One West Australian (WA) study (Hillman *et al.* 2017b) showed that quenda (*Isodon obesulus fusciventer*) were able to be infected with *T. gondii* and survive to mount an antibody response with a 3.7% prevalence. A more recent study of southern brown bandicoots from Kangaroo Island,

South Australia (SA) found a seroprevalence of 0% (=6) (Taggart 2019). However, cross-sectional seroprevalence studies alone are unlikely to be a reliable indicator of level of exposure to toxoplasmosis in marsupials. The use of the Modified Agglutination Test (MAT) will miss identifying individuals in the early stage of infection when only Immunoglobulin (Ig) M antibodies are present. In addition, seroprevalence studies do not reflect the percentage of animals which may die acutely prior to producing any antibodies and hence do not provide sufficient evidence to confirm the role of *T. gondii* in population declines (Hillman *et al.* 2016). Therefore, additional strategies are needed.

Potential strategies for the investigation of pathogen availability in the environment include direct sampling, such as sampling of water and soil (Gotteland *et al.* 2014; Lélou *et al.* 2011), or indirect methods such as measuring prevalence of infection in sentinel species (Anderson *et al.* 2017). There have only been a handful of studies conducted that directly measure *T. gondii* prevalence in soils. The most reliable methods used a gradient diffusion technique to isolate oocysts, followed by molecular polymerase chain reaction (PCR) to identify *T. gondii* DNA (Escotte-Binet *et al.* 2019; Lélou *et al.* 2011; Lélou *et al.* 2012). Some researchers have used methods enumerating oocyst numbers in domestic feline faeces combined with cat density studies to determine *T. gondii* environmental oocyst burdens (Dabritz *et al.* 2007). However, in Australia, this method would rely on access to faeces of free-roaming cats, which, although theoretically possible through the use of detection dogs, is logistically difficult in wild situations.

While soil measurement of *T. gondii* oocysts may give a good indication of the number of oocysts in the environment, it may not accurately inform about the risk of small ground dwelling mammals actually ingesting them. Hence, the identification of a suitable indicator species, which inhabits the same environment and has a similar exposure to *T. gondii* oocysts as the target species may better inform risk of infection with *T. gondii*. The ideal attributes of such a sentinel species were discussed in Chapter One. In summary, a sentinel species should be resistant to developing clinical toxoplasmosis after infection; relatively abundant and easily harvestable; have a relatively short lifespan and small territorial range; live in a similar ecological niche to the target

species and have a similar risk of ingesting oocysts. Two species that fit these criteria in relation to southern brown bandicoots, are the introduced European rabbit (*Oryctolagus cuniculus*) and the domestic house mouse (*Mus musculus*).

Aim of this study

This study aims to investigate methods to assess the probability of infection with *T. gondii* in a small herbivorous marsupial. This will be achieved by measuring the density of oocysts in soil, determining the prevalence of infection in two potential sentinel species (rabbits and mice) and comparing this to the seroprevalence of IgG antibodies to *T. gondii* in an endangered sympatric intermediate host (southern brown bandicoot). In addition, this study aims to continue to investigate the role *T. gondii* may play in the health of the southern brown bandicoot, through measuring seroprevalence of antibodies to *T. gondii* and health parameters (mass, body condition, abundance, ectoparasites) in two populations at sites with and without the presence of cats.

3.2 Methods

Study sites

Two study sites were selected based on the presence or absence of free roaming cats and the presence of free-ranging southern brown bandicoots, mice and rabbits (Nicholls *et al.* 2018) (Figure 3.1). Study Site A, the Royal Botanic Gardens Cranbourne (RBGC) (GPS -38.127307, 145.285873), is approximately 70 km south-east of Melbourne and consists of 380 hectares of remnant bush, mostly coastal heathy woodland with manna gums (*Eucalyptus viminalis*) (Maclagan *et al.* 2018), as well as a central formal, irrigated native garden (Figure 3.3). Study Site B represents three broad areas outside the RBGC. These are: i) an area with a radius of up to 1 km immediately surrounding RBGC, including the Settler's Run Golf Course, roadside verges and local farms; ii) 'Harewood', a private, coastal, rural property west of Tooradin (-38.213357, 145.431655) and iii) rural roadsides and drains in the district formerly known as the 'Koo Wee Rup swamp' surrounding the townships of Koo Wee Rup (KWR)

(-38.193288, 145.488764) and Bayles (-38.177209, 145.568017). The roadside vegetation consists of poor-quality, remnant, native vegetation and includes *Leptospermum* spp; *Eucalypt* spp; bracken (*Pteridium esculentum*) understory; introduced blackberries (*Rubus fruticosus* aggregate) and a range of escaped exotic garden weeds (Maclagan *et al.* 2018) (Figure 3.3).

Cat Density

Cat abundance was likely to differ between sites but estimating cat density was not within the scope of this project. Study Site A has a predator exclusion fence and, while the two public access gates are open from 9am-5pm each day, there has only been the occasional sighting of a cat, which has then been trapped and removed. Regular surveys with spotlights and trail cameras have not recorded any cats at the site in recent years (Coates, T. 2019 pers. comm.) (Nicholls *et al.* 2018). Free-ranging cats are known to occur in Study Site B (Nicholls *et al.* 2018). The density of cats at Site B is not known, however, it is likely to be lower than the 3.4 cats/km² estimated at nearby Phillip Island (Legge *et al.* 2017) as cat density on islands is typically higher than the mainland (Taggart *et al.* 2019). This cat population would likely consist of both feral cats and domestic cats associated with human dwellings in the nearby townships and rural properties.

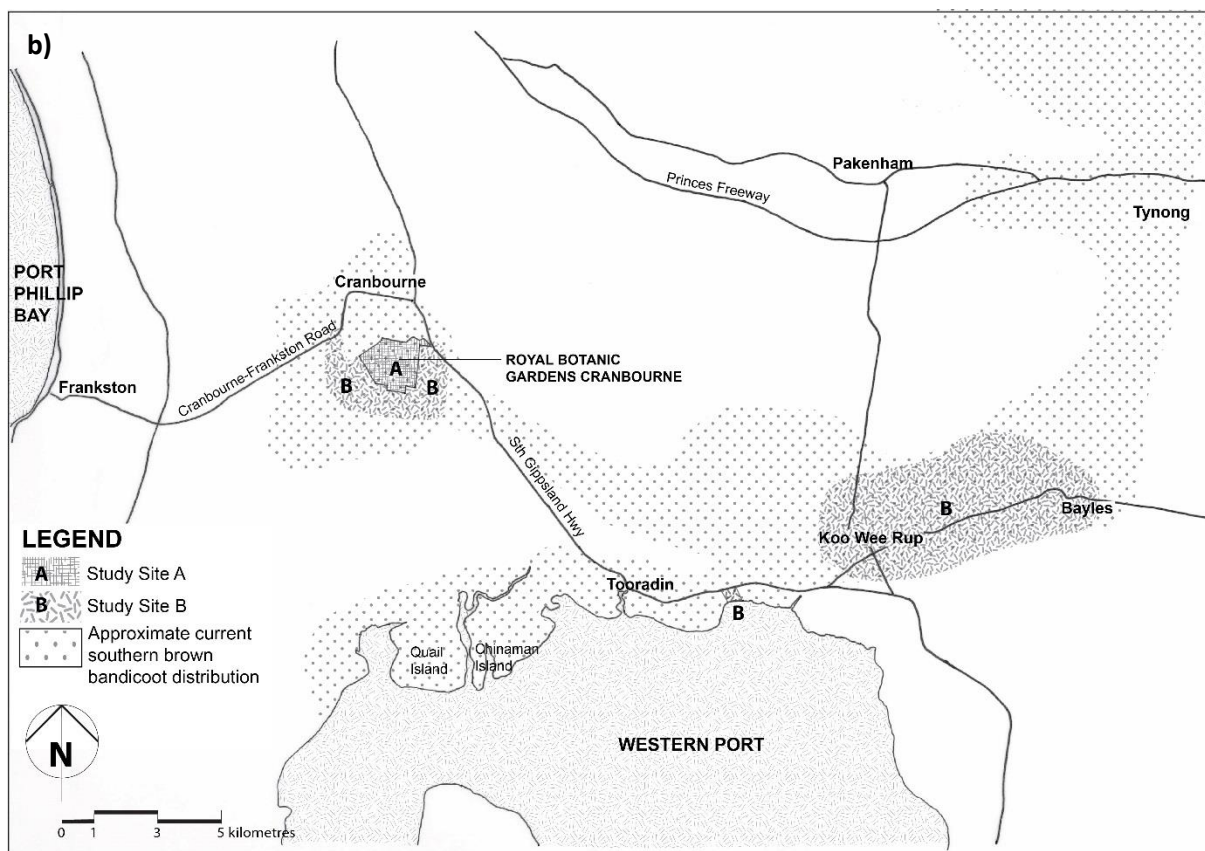
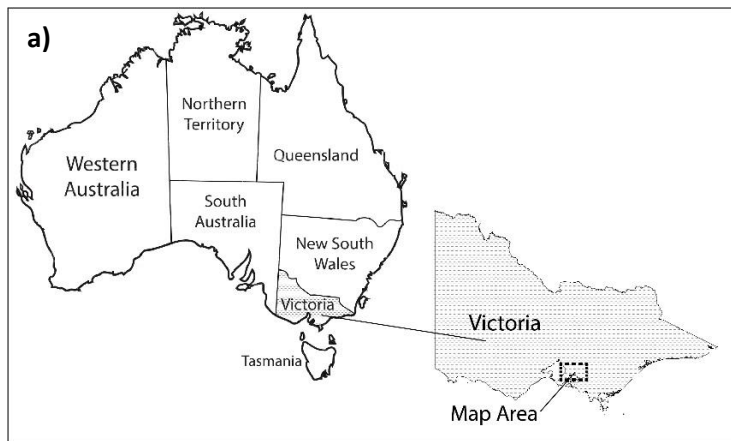


Figure 3.1. Maps showing a) position of study sites in Australia and b) approximate current distribution of southern brown bandicoot (*Isodon obesulus obesulus*) in south central Victoria and the two research study sites.

Collection or capture of study animals

Rabbits

Rabbits were sourced opportunistically from feral pest control programs from both study sites over the period from July 2016 until February 2018. Exact GPS locations of where rabbit carcasses were sourced were not available, however, road-side addresses were recorded. Rabbit heads were collected, triple bagged, labelled and stored at -20°C within a few hours of death. They were later thawed and both eyes, the tongue, 10g of skeletal muscle and the entire brain were dissected from the carcass and placed in a sterile container which was then re-frozen at -20°C, awaiting future DNA extraction and qPCR.

Southern brown bandicoots and mice

Trapping for live southern brown bandicoot and mice occurred at Study Site A on eight nights between March 6th and March 17th, 2017 and at Study Site B on 16 nights between March 27th and April 28th, 2017. This period was selected to avoid the breeding season of southern brown bandicoots, and thus minimise the risk of pouch young being ejected during capture, handling and release (Department of Environment and Conservation 2009; Jackson 2003a). Traps were set at 50 m intervals on both sides of access tracks in the bushland at Study Site A, as well as within the formal gardens on one night. At Study Site B, traps were set in known southern brown bandicoot habitat (MacLagan *et al.* 2018) at 25 m intervals along Railway Road North, Koo Wee Rup and Boundary Drain Road, between Koo Wee Rup and Bayles (see Figure 3.2). GPS positions of traps were recorded. Sixteen further traps were set at 'Harewood' approximately 50 m apart around the homestead garden and along pathways bordering restored natural habitat. At each trap site, one wire mesh cage trap (500 X 250 X 350mm) and two collapsible aluminium Elliott traps (150 x 160 x 450 mm) were placed in concealed positions under low vegetation cover at right angles to runways (tracks made by small animals in undergrowth) with the openings facing the runway (Tasker and Dickman 2001). Traps were baited with a rolled oat and peanut butter mixture (Paull *et al.* 2011) and set prior to dusk. They were checked and emptied the following morning, beginning before dawn. Any non-target species were identified and immediately released. Traps

were left closed during the day and re-set and opened prior to dusk in the late afternoon. All traps had protective waterproof covers and were left in the same location for four days, regardless of trapping success (Tasker and Dickman 2001). Traps were thoroughly washed and sterilised with 10% bleach prior to being moved to new positions each week to prevent any possible transfer of soil, fauna or flora pathogens such as *Phytophthora* fungus, which is known to occur in south eastern Victoria (Newell 1998).

All trapped mice were immediately and humanely euthanised on site, using inhalant isoflurane anaesthetic. They were then sealed in labelled zip lock bags and either dissected on the same day or frozen at -20°C for later tissue dissection. Tissues collected from mice included: brain, heart, lung, liver, kidney, spleen and all skeletal muscle from both hind legs. Those mice that were dissected fresh had approximately 50 µl free blood sampled from the chest cavity, which was stored on a Whatman Filter Paper 903 Protein Saver Card (GE Healthcare Ltd, Cardiff, UK) for possible testing for antibodies to *T. gondii* using MAT, should any mice be found to be positive on qPCR. These cards were stored at -20°C with a silica gel pouch in a sealed zip lock bag.

Southern brown bandicoots were transferred from the trap into a labelled calico bag, weighed using field spring hand-held scales (Salter Brecknell), checked for injuries, any evidence of clinical disease and the presence of a Passive Integrated Transponder (to avoid sampling from re-captures). Those weighing less than 400g but no longer pouch young, were classified as juveniles (Booth *et al.* 1995) and were microchipped on site for future identification with a Passive Integrated Transponder (Trovan Pty. Ltd) placed subcutaneously on the dorsum, between the shoulder blades and then released. The remainder were placed inside individual, labelled pet transport boxes (within their calico bag) and transported in an air-conditioned vehicle to a central processing site within the RBGC at Study Site A or to a private veterinary clinic centrally located at Study Site B. They were given time to recover from the stress of initial capture and handling in a quiet, darkened room.

The southern brown bandicoots were weighed again using precision spring scales (Pesola, Schindellegi, Switzerland) and anaesthetised using an adapted face mask, 5% isoflurane and 2L oxygen/minute, which was reduced to a maintenance rate of 1.5% isoflurane once induction was achieved. A general clinical assessment was made of body condition and general health and any other injuries not detected when conscious. The sex, maturity, morphometric measurements and presence of pouch young were recorded. Subjective body condition was scored out of 5 (see Table 2.1) by the same observer, based on the amount of muscle mass and presence of subcutaneous fat, over the scapular spine, thoracic spine, lumbar spine, pelvic region and hind limbs, similar to that described by Hillman et al (2017). Objective body condition scores were also calculated using a mass to pes length ratio (MacLagan *et al.* 2018). A Passive Integrated Transponder (Trovan Pty. Ltd) microchip was inserted into the subcutaneous space between the shoulder blades, using the producer-supplied applicator. Ectoparasites were opportunistically removed using fine toothed forceps and stored in 70% ethanol. Between 1 to 2 ml of blood was withdrawn from the femoral vein using a 25 G needle. Bandicoots were then allowed to recover from the anaesthetic and placed back in their transport box, with shredded paper and some food (diced carrot and dog kibble) in a warm, quiet room. They were released in the late afternoon at the site of capture.

Two blood smears were made for each animal, fixed with methanol and stained with Giemsa. These blood films were later examined by expert pathologists for the presence of haemoparasites. One drop of blood was placed in an Eppendorf tube, which was frozen for future sequencing, should any haemoparasites be found on the smears. Another 50µl of fresh blood was dispensed onto the centre circle of a Whatman Filter Paper 903 Protein Saver Card (GE Healthcare Ltd, Cardiff, UK), labelled and allowed to air dry completely before sealing in a zip lock bag with silica gel and stored at -20°C. The remainder of the blood was placed into labelled serum separator tubes (BD Vacutainer, Franklin Lakes, New Jersey) and left at room temperature to clot. Once clotted, the tube was centrifuged for three minutes at 3,600 rpm and the serum was removed using a Pasteur pipette. The serum was then apportioned into 0.5 ml aliquots into Eppendorf tubes and frozen at -20°C, awaiting future testing for the presence of antibodies to *T. gondii* using MAT.

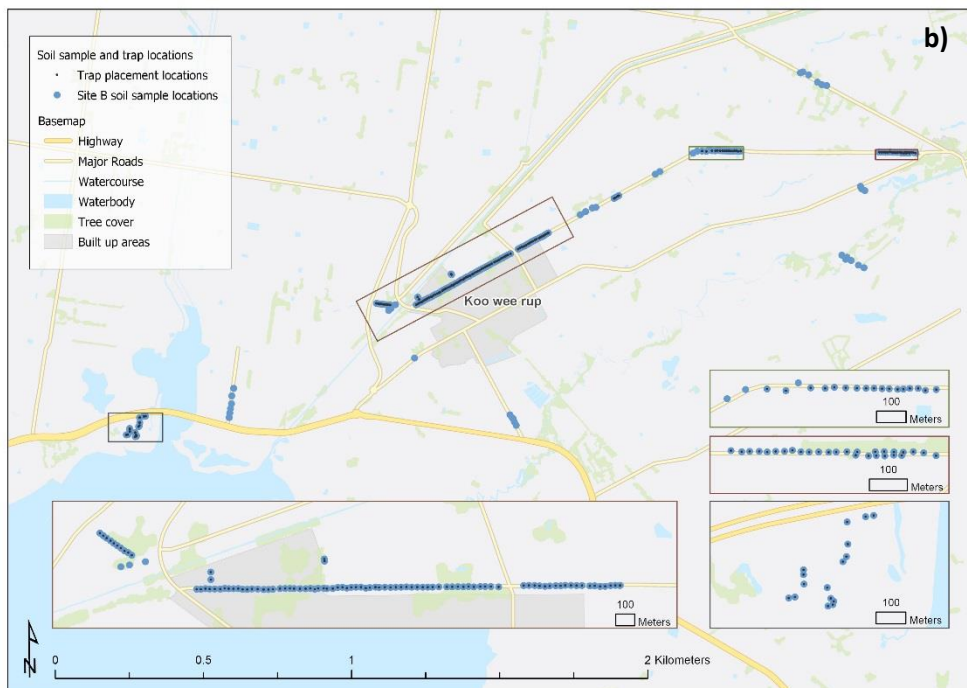
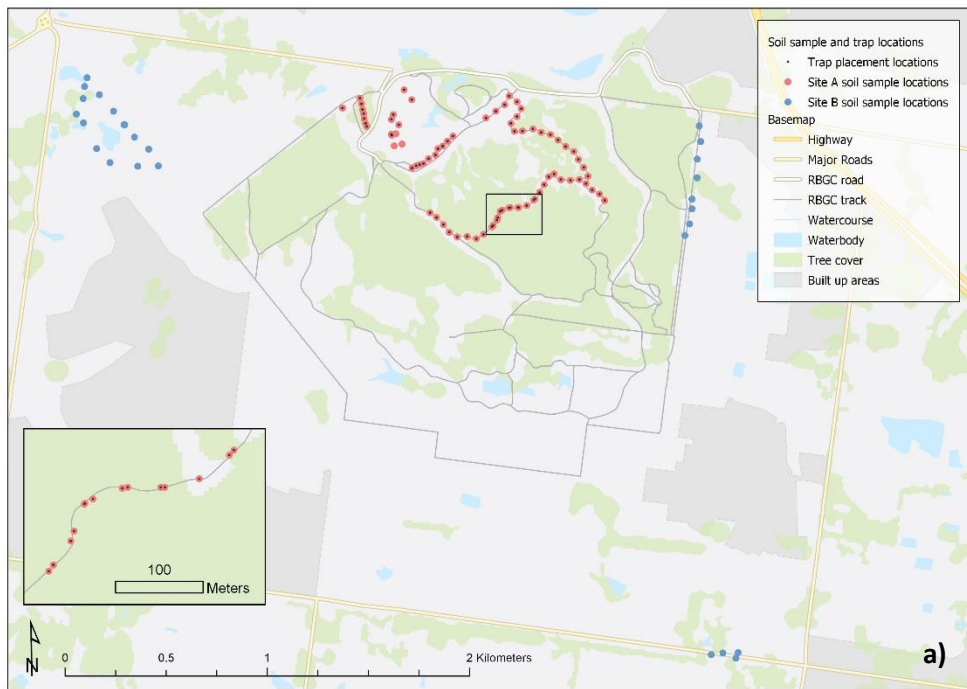


Figure 3.2 Sites of trap placement for southern brown bandicoots (*Isodon obesulus obesulus*) and *Mus musculus* and soil collection sites. a) Study Site A: Royal Botanic Gardens Cranbourne and some of Study Site B outside the RBGC. b) Remainder of Study Site B, showing trap placement along Railway Rd, Koo Wee Rup; Boundary Drain Rd, from Koo Wee Rup to Bayles and at 'Harewood', Tooradin in the east.



Figure 3.3 Habitat at study sites A (Royal Botanic Gardens Cranbourne (RBGC)) and B (Cranbourne, outside RBGC, Koo Wee Rup, Tooradin and Bayles regions). 1) Site B – typical farmland in Koo Wee Rup where rabbits were sourced; 2) Site A – RBGC bushland; 3) Site B - roadside vegetation Bayles; 4) Site A – RBGC Formal gardens; 5) and 6) roadside vegetation Koo Wee Rup

Modified Agglutination Test

The MAT was run according to the modifications used by the National Association of Testing Authority (NATA) – accredited Mt. Pleasant Laboratories (Department of Primary Industries, Water and Environment), Launceston, Tasmania, using the Toxo Screen DA kit (Biomérieux, Marcy-l'Étoile, France) (BioMérieux 2015). For each individual sample 25 µl of serum was serially diluted with phosphate-buffered saline (PBS) to concentrations of 1/8, 1/32 and 1/128. In addition, manufacturer supplied negative and positive controls were diluted with PBS to a 1/8 solution. Subsequently, 25 µl of each sample concentration, as well as the positive and negative controls, were transferred to a round bottom microtiter plate and 25 µl of a 0.2 mol/L solution of mercaptoethanol were added to all sample wells, in order to inactivate any IgM present. After mixing and discarding 25 µl from each of the sample wells, dilutions were 1/16, 1/64, 1/256 (the positive and negative controls were 1/16). Finally, *Toxoplasma* antigen (formalin-treated) was diluted 1:4 with a coloured bovine-albumin-borate saline (BABS) (red) (both supplied with the kit) and 25 µl of the solution added to each of the sample wells. A plate shaker was used to ensure proper mixing of the sample and the antigen, after which the microtiter plate was placed into a closed zip-lock bag on a vibration free bench at room temperature, and results were read after four and six hours. A negative result was interpreted when there was sedimentation representing a small button in the bottom of the well or partial agglutination covering less than half of the well base. A positive result was recorded when there was an agglutination mat covering more than half of the well base.

The blood stored on Whatman's filter paper was used to compare the efficacy of this field collection method for future MAT tests. This test was performed at the Mt Pleasant Laboratories, Tasmania. The central 10 mm diameter blood soaked blotting paper from eight randomly selected samples were each soaked in 350 µl of PBS, being approximately a 1/8 dilution. Assuming half the blood is serum, this is an equivalent of 1/16 dilution, which then became a 1/32 dilution equivalent after the mercaptoethanol was added. The MAT process was then followed as per that described for the serum above. A similar process has been described, which used 300 µl of PBS buffer to soak

one cm² of blotting paper at room temperature overnight (Simon, J.A. 2017. Pers. comm.).

Ectoparasites

Fleas and mites (initially stored in 70% ethanol) were each placed in a drop of lactophenol under a coverslip on a microscope slide and allowed to clear over at least 48 hours. They were then examined under an Olympus BH2 microscope (Tokyo, Japan) and identified to species level (where possible) using taxonomic references for mesostigmatid mites (Domrow 1987) and fleas (Dunnet and Mardon 1974). Ticks were stored in 70% ethanol and later examined under a Nikon SM2 745 dissecting microscope (Tokyo, Japan) and identified to species level (where possible) using taxonomic reference descriptions (Laan *et al.* 2011; Roberts 1969; Roberts 1970).

Mammalian DNA extraction and qPCR

Tissue samples from the necropsied rabbits and mice were thawed. For rabbits, 2 g of brain, 1 g of eye retinal tissue, 1 g of tongue and 1 g of skeletal muscle were pooled. For mice, all collected tissues were pooled and weighed. Tris - EDTA lysis buffer (40 mM Tris, 10mM EDTA – Sigma Aldrich) was added to the tissues at the ratio of 10ml/ 5 g of tissue. The protocol was then followed as described for the mammalian tissue digestion and DNA extraction in Chapter Two. Real time qPCR was performed on the DNA extracted from the mouse and rabbit tissue using the same protocol as described in qPCR Methods of Chapter Two.

Soil Sampling

Soil sampling occurred during the first two weeks of September 2017, when the ground was damp from winter and spring rains. Soil samples were taken from a total of 297 sites. Samples were taken within 10 m of every trap GPS position at both study sites (Figure 3.2). Further soil samples were taken from random locations along the roadside verges corresponding to where rabbits were sourced (Figure 3.2). At each sampling site, five soil samples were taken from five relatively evenly spaced spots within a one-meter

square area, using a cylindrical core 25 mm wide x 50 mm deep. Samples were placed inside ziplock bags, which were double bagged, labelled and stored in the dark at 4° C. All samples remained moist and were processed within three months of collection.

Oocyst recovery

The oocyst recovery protocol was adapted from Gotteland et al (2014) and Lélou et al. (2011) and had previously been validated in our laboratory (Adriaanse 2018). The technique departed from Lélou et al (2011) in that it did not include freeze/thaw cycles to rupture the oocysts, but a mechanical bead beater. The soil inside the collection bag was thoroughly mixed using a spatula and two aliquots, weighing 5 g each, removed and labelled A and B. Each aliquot was subsequently added to 10 ml of de-ionised water in a 50 ml conical centrifuge tube and shaken and vortexed for 2-3 seconds. The soil mixture was overlaid on 20 ml of cold (4°C) sucrose solution (SG 1.23) in a clean 50 ml centrifuge tube and centrifuged at 1500 x g for 20 minutes. Approximately 15 ml of the interface was transferred into a new tube and de-ionised water was added to bring the total volume to 50 ml. This was centrifuged at 1500 x g for 20 minutes. Following this, 49 ml of the supernatant was removed and the remaining 1 ml sediment was collected and stored at -20°C in a 1.5 ml micro-centrifuge tube. DNA was later extracted from 200-300 mg of sediment using the Bioline Isolate Faecal DNA kit (Bioline, London, UK). To ensure oocyst rupture, the re-suspended sediment was agitated with glass beads for 20 minutes at 2700rpm in a tray attached to a vortex agitator (VM1, Ratek, Melbourne). The remaining procedure followed the kit protocol except that DNA was eluted in 50µl of buffer instead of 200µl, as low concentrations of *T. gondii* DNA were expected, and was incubated at room temperature for five minutes. The 50 µl of eluted DNA was frozen in new micro-Eppendorf tubes at -20 °C.

Soil qPCR

The qPCR performed on the DNA extracted from the soil followed the same protocol as used for the mammalian qPCR (Chapter Two), with the exception that instead of the mammalian primers and probes, the qPCR assay included primers and probes specific for the amplification of eukaryotic DNA as an isolation and DNA extraction control.

These Forward and Reverse primers 5' GCC CTA TCA ACT TTC GAT GGT AG 3' and 5' CTA CCA CAT CCA AGG AAG GC 3', (450nM), and probe 5'CGG AGA GGG AGC CTG AGA AAC G 3', (250nM) were added to the final mastermix and optimised to run as a multiplex assay with *T. gondii* and EHV as the target and internal qPCR control respectively. The cycling conditions and analysis was the same as for the mammalian qPCRs. The cut off level of Cq37 was found to be the highest cycling level to detect the presence of *T. gondii* DNA at a dilution of 0.000001pg.

Statistical analysis

Body mass and condition were recorded as a general indication of overall health of the animals and quality of habitat. The results are compared with those of Maclagan (2018), who previously undertook studies of these two populations of southern brown bandicoots, to investigate if there has been a change in these parameters over time. The effects of sex and location (Study Site A – divided into RBGC Formal Garden and RBGC Bush; and Study Site B) on body mass and body condition were examined using linear regression analysis in Minitab 18.1 (Minitab Inc, USA). The structure of the linear models was: Mass ~ sex + location + sex*location and Condition ~ sex + location + sex*location. Histograms of the raw data were visually inspected prior to any analysis to look for any obvious skewing of the data. Body condition was log-transformed to achieve a more normal distribution. After analysis, histograms of residuals were examined to confirm the model assumptions of normal distribution of residuals, plots of residual versus fitted value were used to ascertain homogeneity of variance and a residuals versus order plot was used to test for independence of residuals. Univariable analyses were used to assess association between each predictor (sex and location) and body mass or body condition, followed by building multivariable models including both predictors as well as an interaction between location and sex. In the final models, only those factors significant at the $\alpha = 0.05$ level were included.

3.3 Results

Capture success

Across the two study sites, 29 individual southern brown bandicoots were trapped over 989 trap nights (representing a trapping success of 2.9%). At Study Site A, a total of 15 individual southern brown bandicoots were captured (8 males and 7 females), 13 of which were suitable for sampling. Eight bandicoots (7 males and 1 female) were captured over 411 trap nights in remnant, natural bushland within the site (1.95% trapping success), and seven (6 females and 1 male) on one night (14 trap nights, 50% trapping success) in the central, irrigated formal gardens of RBGC. Two of these individuals were juveniles and hence were microchipped and released without blood sampling. Four bandicoots were captured more than once. Three mice were trapped and euthanised and 11 dead rabbits were obtained (Table 3.1). Several non-target species were also trapped and released at Study Site A. These included two common ringtail possums (*Pseudocheirus peregrinus*), a common brushtail possum (*Trichosurus vulpecula*), a European rabbit and three native swamp rats (*Rattus lutreolus*).

At Study Site B, fourteen individual southern brown bandicoots (3 males and 10 females; 1 unknown sex) were captured over 574 trap nights (2.4% trapping success), 11 of which were suitable for sampling. One adult bandicoot escaped without being microchipped or sampled. Two juveniles were microchipped and released without blood sampling. Eleven were anaesthetised and had blood samples taken; four of these were subsequently re-captured (but only anaesthetised and sampled once). In addition, 264 mice were captured and euthanised and 107 dead rabbits were obtained (Table 3.1). The non-target species which were trapped and subsequently released were 108 black rats (*Rattus rattus*) and 62 native swamp rats.

Table 3.1. Trapping effort and numbers of mice and rabbits captured (all of which were sampled) and of southern brown bandicoots captured (not including re-captures of individuals) and sampled at Study Site A (Royal Botanic Gardens Cranbourne) and Study Site B (Cranbourne, outside RBGC, Koo Wee Rup, Tooradin and Bayles regions).

	Cage Trap	Elliot Trap	SBB	SBB	Mice	Rabbits
	nights	nights	trapped	sampled		
Site A	415	805	15	13	3	11
Site B	574	1148	14	11	264	107

Mass and body condition

The average body mass of adult male SBBs was 685.7g (standard deviation $\pm$ 141.16g; range: 400 - 880g) and 957.5g ($\pm$ 215.46g; 925 – 990g) at Study Sites A and B, respectively. In adult females, this was 601.6 g ($\pm$ 94.53; 480 – 650g) and 741.6 g ($\pm$ 167.02g; 525- 900g). Eight animals were captured more than once, often in different traps. Their first recorded mass and body condition was used to determine the average body mass and body condition statistics. Four of these lost no or minimal body mass between captures and four animals either lost or gained body mass between capture events (see Table 3.2). Subjective body scores ranged from 3 to 5 (Site A) and 2 to 5 (Site B), with a median of 4 at both sites and mean scores of 3.76 and 3.96, respectively.

Table 3.2 Body mass change (g) over repeated captures of southern brown bandicoots (*Isodon obesulus obesulus*) compared to body mass at first capture. Days since previous capture in brackets.

Bandicoot ID	Initial capture weight	1 st re-capture	2 nd re-capture	3 rd re-capture
B2	625	0 (2)	+5 (6)	
B5	880	-5 (1)		
B6	875	-100 (1)	-100 (1)	-75 (1)
B7	275	-65 (1)		
B26	700	0 (1)	-50 (2)	
B30	900	-30 (2)		
B32	650	0 (2)		
B34	800	+75 (1)		

Objective body condition scores, as measured by mass : pes length ratio, showed higher scores in both males and females at Study Site B compared to Study Site A. At Study Site A, those animals captured in the natural remnant bush area showed higher scores than those trapped in the formal gardens (see Table 3.3) but these results were still lower than those at Study Site B.

Table 3.3 Average objective body condition score of adult trapped southern brown bandicoots – based on mass (g) to pes (mm) ratio (MacLagan *et al.* 2018) and separated by sex. Standard deviation in brackets.

Site	males	n	females	n	both sexes
Site A: Bush	13.06 (± 3.07)	5	12.52	1	12.97 (± 2.76)
Site A: Gardens	10.79 (± 1.82)	2	11.17 (± 1.58)	5	10.85 (± 1.67)
Site A: Total	11.88 (± 2.50)	7	11.06 (± 1.66)	6	11.83 (± 2.40)
Site B	13.60 (± 3.87)	9	13.68 (± 2.97)	2	13.88 (± 2.97)
Total combined	12.27 (± 2.71)	16	12.70 (± 2.83)	8	12.77 (± 2.82)

There was no significant effect of sex on body mass or objective body condition (Table 3.4), and no significant interaction between location and sex. However, there was a significant difference based on location. Bandicoots captured at Study Site B were significantly heavier ($p = 0.007$) and in better body condition ($p = 0.036$) than those captured in the RBCG Formal Gardens at Study Site A. Although the trend was for bandicoots from the Bush section of the RBGC to also be lighter than those from Study Site B, this difference was not statistically significant. There was no statistically significant difference in body mass or body condition between bandicoots from the two sections of the RBGC (Study Site A) either.

Table 3.4 Linear regression models for effects of sex and location on body mass (g) and (log-transformed) objective body condition score of southern brown bandicoots (*Isodon obesulus*) captured from two sites in the Royal Botanic Gardens Cranbourne (Site A: Gardens and Bush) and the Koo Wee Rup region (Site B). n = sample size; SE – standard error of coefficient. Significant p-values in bold; identical superscript letters indicate no significant difference

	n	Coefficient	SE	p-value
Body mass (g)				
Intersect	24	747.1	46.2	
Location				
Site B ^A		0	0	
Site A: Bush ^{A,B}		-143.8	88.6	0.120
Site A: Gardens ^B		-214.5	71.2	0.007
Sex				
Female		0	0	
Male		96.0	74.2	0.210
BCI (log-transformed)				
Intersect	24	1.1254	0.030	
Location				
Site B ^A		0	0	
Site A: Bush ^{A,B}		-0.051	0.058	0.389
Site A: Gardens ^B		-0.104	0.047	0.036
Sex				
Female		0	0	
Male		0.035	0.048	0.475

***Toxoplasma gondii* testing**

All 13 southern brown bandicoot blood samples from Site A were negative for IgG antibodies to *T. gondii* as were all 11 samples from Site B (Table 3.5). The replicated blood samples (n=8) from the Whatman filter papers were also negative. This gave an apparent seroprevalence of 0% (95% confidence interval 0-14.25%). Extracted DNA

from tissues of 267 mice underwent qPCR. All results were negative resulting in an apparent prevalence of 0% (95% confidence interval 0 - 1.37%) (Table 3.5). All qPCR testing performed on DNA tissue of 118 rabbits was negative, except for one rabbit (R44, sourced in April 2017 from Sybella Road, Koo Wee Rup, near the Koo Wee Rup sewerage treatment plant) (Table 3.5). This gave an apparent prevalence of *T. gondii* infection in rabbits of 0.85% (1/118) (95% confidence interval 0 - 4.63%) over both study sites. As no mice were positive on qPCR in this study, the MAT test was not performed on the blood samples collected on filter paper to ascertain if vertical transmission was occurring.

In total, 594 soil aliquots were tested from 296 sites, all of which tested negative for *T. gondii* DNA giving an apparent prevalence of 0% (95% confidence interval 0 - 0.62%) (Table 3.5).

Table 3.5 Seroprevalence (Modified Agglutination Test - MAT) and prevalence (qPCR) of *Toxoplasma gondii* in tested southern brown bandicoot (SBB) (MAT), rabbit (qPCR), mouse (qPCR) and soil samples (qPCR). The 95% upper confidence limit is indicated in brackets after the prevalence value. n = sample size

	SBB % (CL)	Rabbit % (CL)	Mouse % (CL)	Soil % (CL)
Study Site A	0 (22.81%) n = 13	0 (25.88%) n = 11	0 (56.15%) n = 3	0 (2.6%) n = 144
Study Site B	0 (25.88%) n = 11	0.93 (5.1%) n = 107	0 (1.43%) n = 264	0 (0.85%) n = 450
Combined	0 (14.25%) n = 24	0.85 (4.63%) n = 118	0 (1.37%) n = 267	0 (0.62%) n = 594

Haemoparasites and ectoparasites

No haemoparasites were detected on southern brown bandicoot blood film slides. Ectoparasites were collected and identified from 19 of the bandicoots (see Table 3.6), with the flea *Pygiopysylla hoplia* and the tick *Ixodes tasmani* being the two most commonly detected. Three different species of fleas were detected: *Pygiopysylla hoplia* was found across both study sites, while *Pygiopysylla zethi* and *Stephanocircus dasyuri* were only found at Study Site A. One bandicoot (B6) had all three species of flea present.

Trombiculid mites were recovered from two individuals captured at Study Site A but specimens were of insufficient quality for identification. *Mesolaelaps antipodanus* was identified from two bandicoots, one from each study site.

The tick *Ixodes tasmani* was only found at Study Site B and *I. hirsti* was identified in the larval and nymph form from one bandicoot each, both from Study Site A. Two bandicoots from Study Site B had nymphs which were either *I. hirsti* or *I. trichosuri*, but currently available descriptions do not allow further differentiation. One bandicoot, trapped in the formal gardens at Study Site A, had a larva and a nymph of (suspected) *Haemaphysalis humerosa*.

Table 3.6 Ectoparasite species found on live-trapped southern brown bandicoots (*Isodon obesulus obesulus*), identified with individual numbers, including minimum prevalence of infestation in last column (%).

	B3	B5	B6	B9	B17	B18	B19A	B19B	B21	B22	B24	B26	B27	B30	B31	B32	B34	B35	B36	%
Fleas																				
<i>Pygiopysylla zethi</i>	•		•			•		•	•											26
<i>Pygiopysylla hoplia</i>			•		•	•		•			•			•	•			•	•	47
<i>Stephanocircus dasyuri</i>			•																	5
Mites																				
<i>Trombiculid sp</i>				•	•															10
<i>Mesolaelaps antipodanus</i>						•										•				10
Ticks																				
<i>Ixodes hirsti</i>				•			•													10
<i>Ixodes hirsti/trichosuri</i>																	•	•		10
<i>Ixodes tasmani</i>										•	•	•	•	•	•	•		•	•	47
<i>Ixodes spp</i>		•						•	•								•	•		21
<i>Haemaphysalis humerosa</i>					•															5

3.4. Discussion

This is the first reported seroprevalence study for IgG antibodies to *T. gondii* in southern brown bandicoots in Victoria. It found no evidence of infection with *T. gondii*, either through clinical signs or the presence of antibodies, in two populations of free-

ranging southern brown bandicoots (n=24) from sites with presumed differing cat densities. The prevalence of infection with *T. gondii* in sympatric rabbits and mice was almost identical to that of sampled bandicoots, and consistent with the concentration of oocysts detected in soil. This supports the hypothesis that testing eutherian species with similar ecological niches may be a valuable tool for predicting the probability of small, ground dwelling marsupials such as the southern brown bandicoot becoming infected with *T. gondii*. However, in the virtual absence of *Toxoplasma*-positive samples (other than the one positive rabbit), it is not possible to confirm a significant association between the prevalence of exposure in the target host (i.e. southern brown bandicoot), sentinels (mouse or rabbit) and soil oocyst concentration at higher levels of contamination.

Southern brown bandicoots

Trapping success

Sample numbers for southern brown bandicoots were lower than expected. The study reflected a similar abundance (3.1%, as determined by trapping success excluding recaptures) at Study Site A as that observed by Maclagan (2018), which recorded a trapping success rate of 0.4 -7.5% in the bushland area of RBGC (Site A). However, Maclagan (2018) recorded a trapping success of 4.9-23.8% (average 6.4%) at 'novel sites' (Site B), whereas this present study only achieved 2.2% in the same locations. There are several possible reasons for this. Firstly, Maclagan (2018) averaged the results over a number of trapping events spread over different seasons in multiple years, whereas this study trapped over just one season in one year and the results may simply reflect seasonal stochasticity. Further, individual bandicoots are known to be more easily trapped after an initial trapping event (Sutherland, D. 2019. Pers comm), and this may have further increased trapping success in the longitudinal study. Secondly, the years of trapping (2012 -2014) in the Maclagan study may have reflected a population rebound after the breaking of the millennium drought of 2000 -2010 where food resources may have become abundant after the scarcity of the drought. Thirdly, bandicoot numbers may be declining as a result of a combination of threats,

including declining quality and fragmentation of habitat, predation and motor vehicle trauma.

Body mass and condition

Although none of the captured bandicoots were positive for infection with *T. gondii*, there was some variation in mass and body condition scores which may be explained by habitat type and quality. Body mass and condition scores were recorded primarily to give an indication of overall health of the bandicoots, particularly in relation to evidence of clinical toxoplasmosis. The average body mass and body condition scores of adults were significantly higher for both males and females at Study Site B compared to the formal gardens at Study Site A. These results suggest that the general health of southern brown bandicoots was poorest in the formal gardens at Study Site A, most likely reflecting poorer quality habitat, including food resources. This is not entirely surprising as the formal gardens are not representative of dense coastal heathland habitat preferred by southern brown bandicoots. Although the trend was for bandicoots from the bush section of Study Site A to also be lighter and have lower body condition scores than those from Study Site B, they were not of statistical significance. This concurs with Maclagan (2018) who found no statistically significant difference in objective body score between modified, 'novel' habitats (such as Site B) compared to remnant bush sites (such as the bush setting in Site A). Maclagan (2018) concluded that southern brown bandicoots in modified sites close to human habitation are likely to benefit from food resources provided to domestic dogs, cats, chooks and horses. In addition, the damp ground associated with the drain system alongside roadways of Koo Wee Rup and Bayles, provides extra food resources favoured by bandicoots, such as arthropods and fungi (Maclagan, S. 2019. Pers. comm.).

Seroprevalence of antibodies to *T. gondii*

The seroprevalence results at Study Site A were consistent with the presumed absence of cats. Although, arguably a bandicoot could become infected with *T. gondii* if it ate picnic scraps from visitors consisting of undercooked meat, containing bradyzoites. However, at Study Site B, an antibody prevalence similar to Hillman (2017) was

anticipated. That study demonstrated a higher seroprevalence of IgG antibodies in quenda from an urban environment (3.7% n=54) compared to those living in bushland habitat (2.9% n=104), with the hypothesis that urban environments have higher densities of cats and hence bandicoots have a greater chance of becoming infected with *T. gondii*. Given that Study Site B in this study included some peri-urban habitat close to the townships and some degraded bushland habitat along roadside verges, a seroprevalence somewhere between these two figures might be expected. However, as the sample numbers at Study Site B were quite low (n=11), the 95% CI range was quite wide (0-25.88%) and did encompass the Hillman et al (2017) results. It is impossible to know whether a sample size closer to that of Hillman et al (2017) would have resulted in a similar seroprevalence. Alternatively, the seroprevalence of 0% may simply be reflecting a low environmental presence of *T. gondii*.

It is highly unlikely that the serology results are false negatives. The MAT tests in this study were checked against negative and positive controls and were conducted strictly according to the modified protocol used by Mt Pleasant Laboratories, Tasmania, who have been using this test for many years (Statham, P. 2017, Pers. comm.). The MAT has not been validated specifically for southern brown bandicoots, however, the kit test has a sensitivity for human serum of 96.2% and specificity 98.8%, which is understood to translate well to marsupial species (Statham, P. 2018 Pers. comm.), with the test being used routinely for marsupial species by researchers (Hillman *et al.* 2017a; Lynch *et al.* 1993a; Obendorf *et al.* 1996). Hillman (2017) calculated a specificity of 93 (79-99) for quenda and reported that they were unable to precisely calculate sensitivity due to insufficient data. Adriaanse (2018) determined that the sensitivity of the MAT in cats was 96.1 % (95 % credible interval 91.5 – 98.8) but the specificity was lower at 82.0 % (65.1 – 93.3).

As discussed earlier, the methodology employed for the present study would not have identified any animals in the initial stages of infection. In addition, this study was unable to detect any southern brown bandicoots that may have died from the acute form of the disease (Wobeser 2007). In a study of 176 brown hares (*Lepus europaeus*), which are

known to be particularly susceptible to acute toxoplasmosis with high mortality, no antibodies (neither IgM nor IgG) were detected using DAT, ELISA, IFAT and Sabin Feldman dye tests (Gustafsson and Uggla 1994). The authors concluded that this provides good evidence that brown hares may be extremely susceptible to acute toxoplasmosis and not surviving long enough to mount an antibody response. This conclusion is also subscribed to by Dubey (2010). The fact that the present study also found a zero prevalence in southern brown bandicoots, could suggest a similar conclusion. However, as this study also found a 0% prevalence of infection in mice and a 0.93% prevalence in rabbits, both of which are generally thought to survive infection (Dubey 2010), across the same landscape, it seems more likely that there was a low risk of infection. If a significant difference in prevalence of infection between sentinel species and the target bandicoot species had been found, then a conclusion that the bandicoots were not surviving infection with *T. gondii* could reasonably be made.

Bandicoots may also have died un-detected from mis-adventure or predation as a result of changed behavioural patterns secondary to infection, as has been shown in rats (House *et al.* 2011) and demonstrated experimentally in eastern barred bandicoots (Bettioli *et al.* 2000a). However, as clinical infection, including possible associated behavioural change, with *T. gondii* has not been investigated in southern brown bandicoots it was outside the scope of this study to take this into account.

The present study included the use of Whatman's Filter paper to validate this method for collecting and storing blood for future field use in detecting serum antibodies to *T. gondii*, as has been the case for cats (Simon, J. A. 2017. Pers. comm.) and other species (Bolais *et al.* 2017). However, without a positive sample, it was impossible to confirm the sensitivity of the method. Several researchers have collected blood from bandicoots and other small mammals from the lateral ear vein, without the need for a general anaesthetic (Bettioli *et al.* 2000a; Fancourt *et al.* 2014; Obendorf *et al.* 1996). The use of filter paper for blood collection and storage that gives a reliable MAT result would thus simplify collection, storage and transport of blood samples from the field.

Ectoparasites

All ectoparasites identified, other than the suspected *H. humerosa*, have previously been reported on southern brown bandicoots in Victoria. This would be the first time *H. humerosa* has been reported on bandicoots on the eastern coast south of Sydney (Roberts 1969; Roberts 1970). Another tick with a similar characteristically elongated body was found on a dead southern brown bandicoot at Study Site A in the same year, however, the head was missing, and it was impossible to categorically identify it to a species level (see Chapter Two). *Haemaphysalis humerosa* was identified from a free-ranging bandicoot from the same population collected (but not reported) several years earlier (Hufschmid, J. 2020. Pers. comm.). It is possible that *H. humerosa* has been present in Victorian southern brown bandicoot communities all along and just not been found and identified. Alternatively, they may have been introduced accidentally, for example on a domestic pet, or its range may be extending southwards in association with climate change.

Ixodes tasmani was only found at Study Site B. Likewise, *P. zethi* and *S. dasyuri* were only found at Study Site A. These species may well be present at both sites, but we simply did not recover any, partly due to our relatively small sample size. Alternatively, these distributions may reflect the fact that the host populations are isolated from each other. Southern brown bandicoot populations have been shown to live in relative isolation and data suggests that the populations at our two study sites are genetically isolated (Coates, T. 2018. unpublished data, Pers. comm.).

Sentinel Species

Except for a single rabbit, the present study found no evidence of infection with *T. gondii* in the sentinel species tested at either of the study sites. These results concur with the soil results and suggest that the environments sampled have a low contamination with *T. gondii*.

The molecular testing methods used in the present study are highly sensitive (99.7% estimate) and specific (94.3%) (Adriaanse 2018), providing confidence in the diagnostic

accuracy. Complete organs and a large percentage of skeletal muscle of the mice were included in the tissue digestion and DNA extraction phase, therefore if any *T. gondii* organisms were present they should have been detected. Equally, as *T. gondii* is regularly found in central nervous system, retinal and skeletal muscle tissue, it is expected that if the rabbits were infected, they would have been identified through the qPCR (Juránková *et al.* 2015).

The low sample size for mice and rabbits at Study Site A affected the study's statistical confidence in this representing a true zero prevalence for those species. The low numbers of mice captured at this site could potentially be explained by failure to attract mice into the traps or be due to a low density of mice. However, mice were caught in large numbers at Study Site B using exactly the same methods, so it seems more likely mice were less abundant at Study Site A, which comprises mostly intact bushland and garden areas. The three mice that were caught were from around buildings in the depot area. Rabbits from Study Site A were sourced from feral control programs conducted inside the RBGC and it was not possible to influence these numbers. At Study Site B, larger sample sizes for rabbits (n = 107) and mice (n = 264) provide greater statistical confidence in the accuracy of these prevalence estimates. This site, was closer to domestic household and farmyard situations, possibly accounting for the higher mouse occupancy and trapping success. The open rural farming environment accounted for the higher rabbit numbers.

The lack of apparent exposure to *T. gondii* at Study Site A, the RBGC, was expected due to the presence of a predator-proof exclusion fence, and lack of cats. It is possible, however, that the rabbits sourced from RBGC also had access to the surrounding farmland, which may have had cats present. At any rate, unless these sentinel species regularly cross the predator-proof fence and are thus exposed to *T. gondii* outside the RBGC, a true zero prevalence for these species appears possible. The very low prevalence of infection with *T. gondii* at Study Site B (0.93% in rabbits, and 0% in mice), on the other hand, was somewhat unexpected, given the assumption that this represented an environment where cats are present.

Environmental loads of oocysts, and likelihood of infection with *T. gondii* has been shown to correlate with cat density (Dabritz *et al.* 2007; Fancourt *et al.* 2014). Dubey (2010) estimates that at any one point in time 1% of cats are shedding oocysts.

Although cat density studies were not performed at Study Site B, cats are known to be present (Maclagan, S. 2019. Pers. comm.) (Nicholls *et al.* 2018) and were observed during the study on a number of occasions. Hence, it is highly likely that cats are shedding some level of oocysts into the environment. It maybe that the estimate of cat density for Study Site B was higher than the reality. Legge *et al.* (2017) reported estimates of cat density on Phillip Island of 3.4/ km² and 8.2/km² in semi-urban areas (Legge *et al.* 2017) but reported lower figures of 0.35, 0.75 and 0.70/km² for East Gippsland, French Island and Wilson's Promontory, respectively. Study Site B reflects a similar topography and human population demographic to Phillip Island, but as islands have been recorded to have higher densities of cats than the mainland (Legge *et al.* 2017; Taggart *et al.* 2019), the density at Study Site B could be significantly lower than 3.4 cats/km² and closer to the values observed in Gippsland, French Island and Wilson's promontory. This could result in relatively low levels of environmental *T. gondii* oocyst contamination, possibly accounting for the difference in prevalence of infection in rabbits in this study compared to Phillip Island (Adriaanse 2018).

It is also possible the cats present at Study Site B were shedding very few *T. gondii* oocysts. Although cats are capable of shedding oocysts at various stages throughout their life and of re-shedding when stressed (Dubey 1976; Dubey 1995b; Dubey 2001), most shedding occurs as kittens (Dubey 2010). Therefore, if a feline population does not contain many young kittens, or if the new infection rate of adult cats is low and if those already infected are not immunosuppressed or otherwise stressed (Dubey 1976; Dubey 1995b; Dubey 2010), there will be relatively low levels of oocyst shed overall. Another factor, which may account for the low measured prevalence in the sentinel species, is that mice and rabbits may not be foraging in areas where free-roaming cats are defaecating and hence not encountering and ingesting infective oocysts.

Study Site B experiences cool, wet seasons over winter, with a mean annual rainfall of 770.5 mm (Bureau of Meteorology 2019) which generally favours *T. gondii* oocyst survival (Dubey 2010). Climatic conditions at Phillip Island, where Adriaanse (2018) found a prevalence of infection of 10.5% (n=134) in rabbits, are similar to those at Study Site B, so climate should not have been a contributing factor to the lower prevalence. However, while rabbit collection took place across several seasons, the mouse trapping occurred after a long, hot summer and dry autumn. While oocysts are known to persist in the environment for more than 18 months (Dubey 2010), some may have been denatured through desiccation, heat and exposure to UV rays (Lélu *et al.* 2012). In addition, many of the mice sampled appeared to be young and hence may not have had an opportunity of ingesting infective oocysts.

The only *T. gondii*-positive rabbit in our study was sourced from the Koo Wee Rup township sewerage treatment plant. It is possible that the sewerage may have been the source of infection for this rabbit, as Cox (1981) similarly concluded that the potential source of higher oocyst availability for rabbits at the Werribee sewerage treatment plant was the sewerage effluent itself, which must contain cat faeces deposited into latrine systems by owners. Alternately, the sewerage treatment plant may simply represent a wetter environment, which favours the persistence of oocysts.

Infection with *T. gondii* in Australian, free-ranging rabbits has previously been reported (Adriaanse 2018; Cox 1981; Harris 2011). While it is considered highly likely that free-ranging mice would also become infected in Australian conditions, this is yet to be demonstrated, with the only two other published studies each reporting a 0% prevalence of infection (Smith and Munday 1965; Taggart 2019). Afonso (2007) cites several pieces of research in the northern hemisphere which show lower prevalence of infection in mice compared with rabbits. The Afonso (2007) research also showed that cats were more likely to become infected with *T. gondii* when they had a higher proportion of rabbits in their diet than mice. It was postulated that the larger size of the rabbit, the increased longevity and wider territory range resulted in greater prevalence of infection in the rabbit compared to the mouse. These factors may also translate to the

southern Australian situation and it may be that mice are not as accurate sentinel species as rabbits.

Soil

No *T. gondii* oocysts were detected in soil samples from either study site. Of interest, Adriaanse (2018) also detected 0% prevalence of oocysts in soil at Phillip Island. The oocyst extraction method from soil, followed by DNA extraction, has been well described by others (Lélu *et al.* 2011; Lélu *et al.* 2012), who successfully extracted oocysts from soil, and was also validated in the laboratory used for this study (Adriaanse 2018). Therefore, if oocysts were present in the samples, it is highly likely that they would have been extracted. It is worth noting that the recovery of soil oocyst technique described by Lélu *et al.* (2011) has recently been refined and includes initially suspending the soil in a 0.1% Tween 80/PBS solution, the use of a 250µm filter bag for 15 minutes and a Fast-Prep mechanical beater to rupture oocysts (rather than freeze/thaw cycles) prior to DNA extraction (Escotte-Binet *et al.* 2019). The present study and that of Adriaanse (2018), had already included the use of glass beads and mechanical agitation to rupture oocysts.

While a 0% prevalence was expected at Study Site A, given the fact that there are no cats present there, a larger prevalence was expected at Study Site B, where cats are known to reside. Cat density and oocyst shedding rates, as just discussed for sentinel species, are the primary determinants of the density of oocysts in the environment, including soil. One explanation is that these results are, in fact, reflecting a true prevalence of oocysts across both sites. However, there may be other factors which influenced this result, including the small sample volumes used in the final testing. Whilst the sample volume collected in the present study was the same as that used by Lélu (2011), the Australian conditions may not have the same density of oocysts as in the French study. A positive result relies on oocysts being present in the final two 5 g aliquots of soil selected for testing. The probability of any particular sample testing positive would thus be significantly reduced if the level of contamination with *T. gondii* oocysts was relatively low to start with, as the results from the sentinel species suggest.

It is also possible that the probability of obtaining positive soil samples was affected by the decision to collect soil based on animal capture sites, rather than using a truly randomised grid or targeting areas likely to have the highest oocyst concentrations, such as cat latrines. A study conducted in France showed that soil oocyst contamination was greater closer to households, dwellings and farms, presumably where cats are thought to be of greatest density, but still persisted beyond cat home ranges (Gotteland *et al.* 2014). Selecting sites within the study area where cats are known to roam and defaecate may increase the likelihood of sampling soil containing oocysts but it would bias prevalence and would not necessarily reflect the movement of oocysts through the environment via water and transport hosts, nor the actual risk of exposure of the hosts of interest at their feeding sites. The exception would be if cats selectively defaecated in sites corresponding to feeding sites of animals under study, however, this behaviour has not been investigated in Australian conditions. Using a randomised grid pattern to collect soil samples may have increased our chance of detecting positive samples, however, whilst this may have been possible at Site A, it would have been impossible at Site B, as sampling was mostly confined to roadside verges. Adriaanse (2018) did collect soil samples (n = 412) on a strictly randomized grid pattern at Summerland Peninsula and Cape Woolamai, Phillip Island, however, no *T. gondii* oocysts were detected in any of those samples either, despite a high seroprevalence in cats of 91.8 % (n = 97). The Adriaanse (2018) results, together with the results presented here, suggest that using randomized soil sampling alone is unlikely to be a reliable indicator of environmental contamination with *T. gondii* oocysts.

Correlation between study results of soil, sentinels and bandicoots

Over two different sites there was a strong correlation between *T. gondii* oocyst prevalence in soil and presence of *T. gondii* DNA in rabbits and mice and the prevalence of antibodies to *T. gondii* in southern brown bandicoots. The consistency of these results across all four measurements, supports the conclusion that the level of environmental contamination with *T. gondii* oocysts was extremely low at the time the two study sites were investigated. These results also support the hypothesis that eutherian species may be able to act as sentinels for the environmental presence of *T. gondii*. However, because the sites investigated appear to have near zero prevalence, it may not be valid

to extrapolate the usefulness of sentinel species across the range of different exposure levels.

3.5 Future research

The design of this study did not allow for the investigation of the incidence of infection with *T. gondii* in southern brown bandicoot populations, nor the outcomes of infection and the effect at the population level. Such studies would require surveys with the ability to track individual bandicoots over extended periods of time, providing longitudinal seroprevalence studies, combined with necropsy and qPCR of recovered dead bandicoots. The inclusion of cat density and cat seroprevalence studies would add value to future research. Research, such as this, will be essential to determining the role *T. gondii* plays in the population health of these threatened marsupials and to design appropriate conservation strategies.

As this current study provided evidence of a possible decline in southern brown bandicoot populations since the research by Maclagan (2018) and supported by Coates (2019, unpublished data) further monitoring of the populations, with particular emphasis on identifying key threatening processes is recommended.

Research is also recommended across a variety of Australian landscapes, in a range of climatic conditions and seasons and at sites with different cat densities, to further investigate the potential of mice and rabbits to act as indicator species to identify the risk of ground-dwelling native mammals becoming infected with *T. gondii*. A component of this is recommended to investigate whether free-ranging mice are in fact becoming infected with *T. gondii* in Australian ecosystems. Such a study should also investigate whether vertical transmission of *T. gondii* is occurring in free-ranging mice in Australia, because if a significant proportion of mouse infections are occurring through vertical transmission, prevalence of infection surveys will not reflect infection acquired from the environment. Future research could also investigate the use of other sentinel species, including bush rats, black rats and invertebrates such as earthworms, which have been

shown to act as paratenic hosts for *T. gondii* oocysts with potential for bioaccumulation (Bettioli *et al.* 2000b).

Further investigation in refining techniques for direct soil analysis in the Australian scenario is recommended, including methods such as sampling greater volumes of soil and using a greater sampling density, to improve the chances of collecting soil containing oocysts.

By pairing filter paper and conventional blood collection, it is recommended that future research further validates the use of filter paper as a method of blood collection from small mammals and storage prior to performing MAT. This would simplify collection and transport of samples for conservation managers in remote areas and require less complex laboratory work.

3.6 Significance of this study

This study represents the first to assess a landscape for environmental *T. gondii* burdens using soil sampling, two eutherian species and a native marsupial. It provides important information about the presence of *T. gondii* in that environment and a potential model for measuring other landscapes for the prevalence of *T. gondii* contamination, for example in preparation for conservation translocations of native mammals. It is the first seroprevalence study for antibodies to *T. gondii* conducted in Victorian southern brown bandicoots and, along with morphometric measurements and ectoparasite identification, adds to our knowledge about this species. Likewise, it is the first Australian study to investigate the prevalence of *T. gondii* infection in free-ranging mice and the first study to investigate if mice are capable of acting as sentinel species for environmental contamination with *T. gondii*. The present study and Adriaanse's (2018) work, are the first two to investigate prevalence of *T. gondii* oocysts in Australian soils and the first to undertake large surveys of prevalence of infection with *T. gondii* in Victorian rabbits since Cox (1981). These studies can form the basis of further investigations of environmental contamination with *T. gondii*.

3.7 Permits

All trapping and animal work adhered to the guidelines provided in Use of Australian Native Mammals in Research and Teaching (NHMRC 2014) and were performed under the University of Melbourne Animal Ethics Committee approval number 1613973.1, the Department of Environment, Land, Water and Planning (DELWP) permit number 10008263 and a RBGC research permit RBGC-1701.

CHAPTER FOUR

GENERAL DISCUSSION

4.1 Introduction

Australia has witnessed more mammalian extinctions than any other country in the last 240 years (Woinarski *et al.* 2015). Small, ground-dwelling mammals, including the endangered southern brown bandicoot (*Isoodon obesulus obesulus*), are vulnerable to threatening processes (McKenzie *et al.* 2007) and conservation efforts are focussed on protecting these remaining species, particularly those listed as endangered under the Australian Environmental Protection and Biodiversity Conservation (EPBC) Act 1999.

Free-ranging populations of the southern brown bandicoot face many threats, including predation and habitat loss (including through wildfire), contraction and fragmentation (Coates *et al.* 2008). Disease may also play a role, however, there has been very little research into the health of southern brown bandicoots in particular, to investigate whether southern brown bandicoots are susceptible to infection with the protozoon parasite *Toxoplasma gondii*, nor the role this parasite may play as a threatening process to individuals and populations.

The overarching aim of this thesis was to investigate the role that *T. gondii* may play in the health of the endangered southern brown bandicoot in free-ranging populations in the northern hinterland of Western Port, Victoria. In addition, methods to assess environmental contamination with *T. gondii* and the probability of infection of small marsupials with *T. gondii* were investigated by using two eutherian species as sentinels and using direct soil oocyst measurements.

To achieve these aims, the thesis:

1. Established the cause of death in a series of southern brown bandicoots through necropsy studies, including determining the prevalence of infection with *T. gondii* using qPCR

2. Determined the seroprevalence of antibodies to *T. gondii*, using the Modified Agglutination Technique (MAT), in a field based cross-sectional study of two free-ranging southern brown bandicoot populations in two sites with differing cat densities, in the coastal heathlands and hinterland of Western Port
3. Statistically analysed morphometric measurements of the southern brown bandicoots trapped in the field study, to provide indicators of health and habitat quality
4. Identified endoparasites and ectoparasites of necropsied and live-trapped southern brown bandicoots
5. Measured prevalence of infection with *T. gondii* in free-ranging populations of mice (*Mus musculus domesticus*) and rabbits (*Oryctolagus cuniculus*) at these same sites
6. Measured prevalence of *T. gondii* oocysts in soil samples from these sites
7. Explored whether free-ranging mice and rabbits can act as suitable indicator species for the environmental presence of *T. gondii* oocysts. Such an indicator species would play a valuable role in informing conservation management of mammals and birds at sites across most of Australia.

4.2 Overview of key findings

In the necropsy series of 33 southern brown bandicoots, several causes of death were identified, including road trauma (22), predation (4), ejected pouch young (2), drowning (1), pyometra (1) and a death suspected to be associated with lentil ingestion (1). In two animals, no cause of death could be identified. Predation and trauma as the predominant cause of death was consistent with the findings of the only other necropsy series reported in southern brown bandicoots from these locations (Jansen 2017). Tissues from 30 of the cases in this necropsy series were tested for the presence of *T. gondii* DNA using qPCR and all were negative. A range of internal and external parasites were collected and identified, all of which are considered to be part of the normal parasitic fauna associated with southern brown bandicoots and not a cause of significant disease (Domrow 1987; Roberts 1970; Spratt and Beveridge 2016).

The field study compared two sites with presumed differing cat densities (Study Site A - presumed zero presence of cats, Study Site B – a known cat presence) and hence presumed differing levels of environmental contamination with *T. gondii*. No evidence of exposure to *T. gondii* was found in any of the soil samples (n=594), southern brown bandicoots (n=24) or potential sentinel species (118 rabbits and 267 mice) examined, excepting one positive qPCR result in a rabbit at Study Site B.

4.3 Implications of these results

These results have addressed the overall aim of the thesis, to identify what role *T. gondii* may be playing in the health of southern brown bandicoots in the geographical area studied. By combining the qPCR and histopathology results from the necropsy series with the MAT results and physical examination (assessing clinical signs such as poor body condition and gastrointestinal, ocular or neurological symptoms) from the live-trapped bandicoots, no evidence of infection with *T. gondii* was found. The environmental evaluation using two sentinel species and soil sampling further concluded that there was a minimal presence of *T. gondii* in the environment and hence a minimal probability of infection for the southern brown bandicoot. These results imply that *T. gondii* in these populations of southern brown bandicoots is unlikely to be a threatening process causing population decline. These results were obtained despite a known cat presence and cool, wet, climatic conditions, which would suggest there would be a measurable presence of *T. gondii* in this environment (Dickson 2018).

Even if *T. gondii* was endemic in southern brown bandicoot populations, it may not result in population declines, provided that recruitment occurs at a rate to compensate for any losses due to decreased fecundity or mortality resulting from infection with *T. gondii*. Eastern barred bandicoot populations persist in the wild in Tasmania, with an endemic *T. gondii* presence (Obendorf *et al.* 1996) as do quenda (*Isodon obesulus fusciventer*) populations around Perth, West Australia (WA) (Hillman *et al.* 2017b). It is unknown whether *T. gondii* affects overall reproductive output in bandicoot species, as occurs in other species, such as sheep (Dubey 2009). If other threatening processes are also negatively impacting population growth, such as poor connectivity between

populations due to isolated or fragmented habitats and high predator densities, then the effect of the disease on population growth may become more significant (Scheele *et al.* 2019).

However, the necropsy series in this thesis did identify motor vehicle trauma (MVT) as the predominant cause of death in those examined and serves to highlight MVT as a significant threat to southern brown bandicoot populations, as has been identified in other species (Dexter 2019; Griffith *et al.* 2013; Grueber *et al.* 2017). It is important that mitigating traffic strategies are implemented both on public roads and within the RBGC. Predation was the second highest cause of death, confirming the impact that cats, foxes and dogs continue to have on critical weight range (CWR) Australian fauna (Murphy *et al.* 2019; Woinarski *et al.* 2017; Woolley *et al.* 2019) and the ongoing necessity of feral predator control for the survival of free-ranging populations of this species (Woinarski *et al.* 2014a) and the importance of maintaining exclusion fences in protected areas.

This thesis has had to reflect on the complexity of interpreting the absence of a disease in the target species, in sentinel species and in soil samples. The difficulty of ascertaining whether or not a disease is actually present in an area and delineating the actual area which sentinel species have covered are issues that have challenged many researchers (Yockney *et al.* 2013). In New Zealand there has been an ongoing eradication campaign for bovine tuberculosis (TB), including culling introduced possums (*Trichosurus vulpecula*), which act as disease reservoir hosts (Nugent *et al.* 2014). As eradication programs have progressed and prevalence of *Mycobacterium bovis*, the causative agent of bovine TB, drops closer to zero in cattle and as possum numbers decrease, a range of surveillance methods have been employed. In order to estimate the remnant environmental contamination with *M. bovis*, feral pigs (*Sus scrofa*) (which scavenge on possum carcasses) have been used as sentinel species (Nugent *et al.* 2014). Other studies have combined results from sentinel cattle and wildlife to enhance the surveillance of *M. bovis* (Anderson *et al.* 2017), while the home ranges of possums, feral pigs, deer (*Cervus elaphus*) and ferrets (*Mustela furo*) have also been measured and used in combination with prevalence studies to more accurately predict remnant areas

of TB in the landscape (Yockney *et al.* 2013). Such models may also be helpful in improving the accuracy of sentinel species indicating environmental contamination with *T. gondii*. By including two sentinel species with small home ranges, a target native species and soil oocyst measurements, this thesis aimed to more accurately predict the environmental contamination, or probability of ingestion of *T. gondii* oocysts than would have occurred by using just one measurement in isolation.

This study demonstrates the value of using several parameters to assess an environment and the potential value of sentinel species, such as mice and rabbits, to inform the probability of infection with *T. gondii* in target species. Apart from one rabbit, there was no evidence of infection with *T. gondii* in the mice (95% CI: 0 - 1.37%) and rabbits (95% CI: 0 - 4.63%) using qPCR on tissues, nor was there any evidence of oocysts present in the soil (95% CI: 0 - 0.62%). This provides greater confidence in concluding that, while the CI for bandicoots was 0 - 14.25% overall and 0 - 25.88% at Study Site B, the absence of *T. gondii* in the southern brown bandicoots is most likely because they are not ingesting infective *T. gondii* oocysts due to a low prevalence of the parasite in the environment, rather than because they are becoming infected and dying undetected from acute toxoplasmosis. If *T. gondii* is present in the landscape, it is likely to be heterogeneous and was not reflected in the sampling in this study.

Prevalence of infection with *T. gondii* in sentinel species should reflect the presence of infective, sporulated oocysts in the environment, as compared to soil sampling, where qPCR can only give information about the presence or absence of *T. gondii* DNA and not infectivity of oocysts. In addition, sentinel species reflect the actual probability of ingestion of an oocyst by a bandicoot, or other small, native mammal, assuming the sentinel species have a similar risk of ingesting *T. gondii* oocysts and feed in the same area. Southern brown bandicoots forage largely through turning soil over and eating arthropods, seeds and fungi (Valentine *et al.* 2012; Valentine *et al.* 2017). This foraging behaviour is partially reflected in mice. As well as seeds and plant material, wild house mice diet includes an average of 9.4% invertebrates (Bomford 1987; Tann *et al.* 1991), some of which presumably are ingested from within soil litter. In Europe,

free-ranging mice have been shown to become naturally infected with *T. gondii* (Hurkova-Hofmannova *et al.* 2014; Krücken *et al.* 2017; Machacova *et al.* 2016). Similarly, rabbits also dig and scratch through the soil and graze vegetation close to the ground, hence would also be inadvertently consuming soil. While rabbits have been shown to become infected with *T. gondii* in free-ranging Australian conditions (Adriaanse 2018; Cox 1981; Harris 2011) with prevalence of infection correlating with cat density and cooler, wetter climates, it is not known if mice are becoming infected, nor if they are capable of reflecting similar information. This is the first Victorian study investigating prevalence of infection with *T. gondii* in free ranging mice and the first Australian study to use qPCR on mouse tissues to identify infection with *T. gondii*. The only two previous Australian mice studies, in western Queensland (n=4), using mouse bio-assay (Smith and Munday 1965) and in South Australia (SA) (n=55), using MAT on sera (Taggart 2019) also found 0% prevalence of infection. It is possible that the life span of mice may be too short to reflect an annual probability of infection. For example, if mice are very young, (as many of the ones trapped in this study appeared to be) and are trapped in late autumn after a long hot summer, there may not be the same concentration of viable oocysts present in the environment, and hence a decreased likelihood of becoming infected. This would result in a low prevalence of infection but may not reflect an overall annual prevalence.

This thesis represents only the second seroprevalence study of IgG antibodies to *T. gondii* in southern brown bandicoots (*Isodon obesulus obesulus*) (Taggart 2019). It is the first to compare two populations where one resides in what is believed to be a cat free zone, Study Site A, (Coates, T. 2019. Pers. comm.) and the other in an area with a known cat presence, Study Site B (Maclagan, S. 2018 pers. comm.) (Nicholls *et al.* 2018). Thus, it is adding to the knowledge bank about disease in this species. The study performed by Adriaanse (2018), along with this study, are the first to test Australian rabbits for the presence of *T. gondii* using qPCR and represent the largest published rabbit surveys for the prevalence of *T. gondii* in Victoria since 1981 (Cox 1981). This thesis and the Adriaanse (2018) study are also the first two studies to specifically investigate if eutherian species can act as indicators to inform about potential *T. gondii* exposure in Australian mammals. In addition, these studies are the first Australian

studies to attempt to isolate *T. gondii* oocysts from soil and use qPCR to test for the prevalence of *T. gondii* DNA in the natural environment (Adriaanse 2018).

This study represents only the second comprehensive report of a necropsy series of southern brown bandicoots from a defined geographical area. All other reports are from isolated, individual southern brown bandicoot necropsies held by the national registry of Wildlife Health Australia (WHA) or necropsies undertaken by Zoos Victoria (Lynch 2010). Combined with the findings of Janson (2017), it provides a significant data set over a time span from January 2011 to February 2018 of southern brown bandicoot necropsy reports, histopathological sections and parasite collections for future reference. They represent the first reports of southern brown bandicoot tissues undergoing PCR (Jansen 2017) and qPCR (Chapter Two) analysis for the presence of *T. gondii* DNA. The necropsy series (Chapter Two) identified a metastrongyloid nematode in the lungs of one of the bandicoots, providing the first reported case of lung worm in a southern brown bandicoot. The necropsy results also revealed a possible range expansion for one ectoparasite. A *Haemaphysalis* sp. (most likely *H. humerosa*) adult tick was found on one dead southern brown bandicoot from the Royal Botanic Gardens Cranbourne (RBGC). A larval and a nymph form of *H. humerosa* were found on one live bandicoot, also from the RBGC (Chapter Three). In addition, *H. humerosa* has recently been identified from a live trapped bandicoot from the same area in an earlier research project (Hufschmid, J. 2020. Pers. comm.). These are the first records of this species south of Sydney (Roberts 1970). The fact that different life stages were found on more than one animal, suggests this species of tick is endemic in this population. The tick may have been previously present but the lack of previous ectoparasite surveys precluded accurate representation of its presence and distribution in the region. Alternatively, it may have extended its range southwards with increased movements of people and animals or in response to climate change, as has been demonstrated with *Dermacentor reticulatus* in Europe (Gray *et al.* 2009; Sréter *et al.* 2005). *Haemaphysalis humerosa* was first collected and described from bandicoot species in WA in 1909 (Roberts 1963) and by 1915 was also identified in the Northern Territory and NSW. By 1948 it was listed as being present on bandicoots in Queensland but not Victoria (Roberts 1963). The primary natural hosts of *H. humerosa* are bandicoot and rodent species but it has also

been identified on a range of avian hosts and an echidna (*Tachyglossus aculeatus*) (Roberts 1963). In Victoria, as well as the southern brown bandicoot, it could therefore possibly use the echidna, the native bush (*Rattus fuscipes*) and swamp (*Rattus lutreolus*) rats and the exotic black (*Rattus rattus*) and brown (*Rattus norvegicus*) rats as hosts.

Haemaphysalis humerosa was identified on quenda in WA, with 5.6% (n=161) of animals trapped in bush habitats infested compared with 0.8% (n=126) trapped in urbanised environments (Hillman *et al.* 2017b). These results suggest that this species of tick is more prevalent in natural bushland and may explain why they were found in the natural bush of RBGC and not in bandicoots trapped on the roadside verges of Study Site B. *Haemaphysalis humerosa* has been shown to be a vector of *Coxiella burnetii* (the haemoparasite responsible for the zoonotic Q fever) (Derrick 1944) and is also a known vector of *Theileria buffeli* (a protozoan haemoparasite of cattle in Queensland responsible for ‘tick fever’) (Stewart *et al.* 1987), hence its presence in Victoria may be of significance to the cattle industry.

4.4 Limitations of the study

Whilst this study has made some significant findings, there are limitations to be considered. To undertake a full investigation of bandicoot health much larger sample numbers would be required of both necropsied and live -trapped animals. In addition, to identify the presence of pathogens listed in Table 1.1, a full health surveillance should include urine analysis (difficult in bandicoots) to determine renal function and identify pathogens such as *Leptospira* spp, *Klossiella quimrensis* and *Chlamydia* spp; full blood and serum analyses to determine health parameters and organ function; examination of blood smears to identify haematozoa species; the development of antibody studies of a range of diseases, including *C. burnetii*; faecal analysis to identify internal helminths, other protozoan species, including coccidia, *Sarcocystis* and *Eimeria* spp; cloacal, nasal /oropharyngeal swabs, culture and PCR to identify the presence of other micro-organisms (including *Chlamydia* spp) and viruses (including herpes species such as Peramelid herpesvirus 1) and swabs of cutaneous lesions to identify superficial bacterial infections and to screen for Bandicoot Papillomatosis Carcinomatosis Virus 1 and 2 (BPCV1) and (BPCV2).

This study was a simple cross-sectional study, which precluded incidence of toxoplasmosis and mortality being assessed. To ascertain the true effect of *T. gondii* on a population, ideally studies should include permanent identification of individuals, telemetry to allow early retrieval and necropsy of dead animals and regular seroprevalence, health and population surveys. This would enable the fate of individuals to be determined and give a better indication of true prevalence of initial infection, whether or not clinical toxoplasmosis ensues, the survival and mortality rates, the effect on fecundity and the overall effect on population demographics. However, telemetry and tracking, particularly of ill and dead animals, is extremely difficult in bandicoot species over extended periods (Sutherland, D. 2019. Pers. comm.). In the case of the two populations in this study, all that can be concluded from the raw prevalence figures from the necropsies and trapping is that there was no evidence of *T. gondii* infection in the southern brown bandicoots that were tested. Given the close to zero prevalence of *T. gondii* in sentinel species and soil samples, it seems unlikely that *T. gondii* is causing significant mortalities and morbidities in these two populations. The same may not be true for other populations, living in more cat dense areas.

The necropsy series reported on the causes of death of a series of bandicoots reported to The University of Melbourne. However, this opportunistic sampling is inherently biased as it only accesses those cadavers that are both obvious to an observer and that someone has gone to the effort to collect and report. Consequently, not all southern brown bandicoots that died in that period were necropsied, as most will have died away from observation by people and possibly been scavenged while others may have been observed but not collected and reported to the university. Bandicoots that have been predated and consumed will also not be included in a necropsy series. Hence, the bodies recovered may be biased towards particular modes of death, for example motor vehicle trauma, where they are easily observed. The majority of the cadavers examined were sourced from RBGC, (see Appendix 2.1), which has a close to zero cat density and therefore necropsy results did not proportionately reflect causes of death or prevalence of infection with *T. gondii* from populations outside of the RBGC. So, while necropsy is a valid and valuable approach to gaining knowledge about health and causes of death in bandicoots, these biases will limit some of the conclusions that can be drawn.

The low sample size of southern brown bandicoots in the live-trapping field work has limited the interpretation of results. In addition, the trapping relied on bandicoots entering traps and hence there was a bias against any bandicoots that may have been ill, including from toxoplasmosis, encountering traps or being inclined to enter them. A larger sample number would have increased the probability of finding individuals with IgG antibodies to *T. gondii* and would have given greater statistical confidence with a smaller range of 95% confidence intervals. It was anticipated that some evidence of *T. gondii* infection in southern brown bandicoots would be detected in Study Site B, similar to that found by Hillman (2017). In fact, the low sample numbers at Study Site B, gave an upper 95% confidence level for seroprevalence was 25.88%, which did encompass the Hillman (2017) results of 2.9% (3/104) from bushland environments and 3.7% (2/54) from urban environments. By including sentinel species in the study, with higher sample numbers, the study was able to mitigate to some degree the small sample size of the southern brown bandicoots to estimate probability of infection and environmental contamination. The study would also have benefited from sampling over different seasons and over a series of years, to account for seasonal and annual variation of conditions.

The MAT used to determine seroprevalence of antibodies to *T. gondii* is limited as it only detects IgG antibodies, which are formed over a period of weeks after initial infection and does not detect not IgM antibodies which are formed in the initial stages of infection (Bettioli *et al.* 2000a). Hence, if a percentage of southern brown bandicoots had been infected during the three weeks prior to trapping and had survived, their exposure to *T. gondii* would not have been detected. Including the Direct Agglutination Test (DAT), which measures IgM, may have in part addressed this, although it is considered not as sensitive (Statham, P. 2018. Pers. comm.). There is a small possibility that some of our results were limited by the sensitivity of the MAT test and we may have had some false negatives, however, this is unlikely given that the kit reports the sensitivity for human sera as 96.2%, which is understood to be similar across species (Statham, P. 2018. Pers. comm.).

It was not within the realms of the study to determine cat density at each site. This does limit some of the conclusions that can be drawn. Measures of cat density would have enabled us to link prevalence of *T. gondii* with cat density as a surrogate for environmental *T. gondii* oocyst loads. If, for example, there was a very low cat density across this study site, then there may have been a low oocyst environmental burden (Dabritz *et al.* 2007; Fancourt and Jackson 2014) which may explain the close to zero prevalence that this study obtained across all species and soil samples. Likewise, the study was unable to measure the proportion of cats that were truly feral compared with those which maybe free-roaming domesticated cats from local towns and farms. While the prevalence of infection has not been shown to differ between urban and feral cats (Sumner and Ackland 1999), there may be other factors to be taken into consideration. Cats belonging to households may have a smaller proportion of kittens, due to de-sexing, and kittens which are born may be prevented from roaming at the time when they are most likely to be shedding oocysts (Dubey 2010). In addition, cats belonging to households are more likely to use indoor kitty litters and less likely to be nutritionally stressed or immune compromised, factors which can lead to re-shedding (Dubey 1976; Dubey 2001; Dubey and Davis 1995). Oocyst shedding in feral cats may also be affected by availability of infective prey, such as mice, which in turn may be influenced by seasonal factors (Afonso *et al.* 2010; Afonso *et al.* 2007; Tann *et al.* 1991).

It would have also been informative to measure seroprevalence of antibodies to *T. gondii* or prevalence of *T. gondii* in tissues by qPCR in the local free-ranging cats, as in Adriaanse (2018), however, this would have been difficult to achieve as Study Site B was across multiple private land holdings and roadside verges. If seroprevalence was low in the cats in Study Site B, then it is possible that the number of oocysts shed into the environment would have been correspondingly low, accounting for the low prevalence of infection found in the study animals. However, seroprevalence on its own is not necessarily a good indication of shedding (Dubey 1995b) and other factors, such as stress, which impact the immune system are involved (Dubey and Davis 1995). Finally, given cat latrines are not homogeneously distributed across the landscape, (Afonso *et al.* 2010), it is also possible that this study missed sites of *T. gondii* oocyst contamination associated with areas of cat activity.

It is possible that season may have affected the results, as heat, desiccation and UV light decrease environmental viability of oocysts (Dubey 1998; Dubey 2010). Trapping in late Autumn after a long, hot dry summer may have correlated with a smaller number of infective oocysts in the environment, leading to a decreased chance for sentinel species to acquire infection, particularly in the case of young mice. If trapping had occurred through the other seasons, especially winter and spring, a greater prevalence of *T. gondii* in these species may have been recorded. Soil sampling, on the other hand was performed in spring, to maximise the possibility of collecting oocysts, when soils were cool and moist. The fact these samples were not collected at the same time as the southern brown bandicoot and mouse trapping was performed, could be viewed as a limiting factor, however, antibodies are present within a bandicoot over several years, and bradyzoite cysts remain in mouse tissues over a lifetime, so it would only have been animals born over the summer period that would have had limited access to *T. gondii*. Regardless of which, oocysts are able to survive in hot dry environments for periods exceeding 18 months, particularly if covered, so many should have survived and remained infective over the summer period (Dubey 2010). The rabbits were sourced gradually over a two-year time frame, which accounted for seasonal variation in availability of infectivity of environmental oocysts. Likewise, the necropsy cadavers were collected at random times over a period of several years and hence were not subject to seasonal bias.

Soil sampling in this study was not carried out as a truly random grid but followed a linear pattern along road sides and tracks sampling at regular intervals corresponding to trap placements, which may not have coincided with cat latrines or areas where oocysts may have been washed or moved by transport hosts. Had soil been sampled in a truly random grid, oocysts may have been detected. However, Adriaanse (2018) used a true randomised grid pattern and did not recover any *T. gondii* DNA from soil samples in nearby Phillip Island, despite the presence of 90.2% seropositive cats with a density of 3.4 cats / km². Of interest, in the two areas where soil samples were tested at Phillip Island, Summerland Peninsula and Cape Woolamai, rabbits seroprevalence was also 0% (n=38 and 2 respectively). A seroprevalence of 21.6% was recorded in rabbits from Cape Kitchen, however, soil sampling did not occur at this site (Adriaanse 2018). Whilst

the laboratory protocol has high sensitivity and specificity, only two aliquots of five grams each of soil were selected from a bag of sampled soil weighing up to 100g, so oocysts may have been present in the sample collected in the field but randomly not included in the 10g that was tested. To remove this risk of missing oocysts, a protocol could possibly be developed to extract all potential oocysts from a much larger volume of soil. Nevertheless, the fact that neither study found any *T. gondii* oocysts over hundreds of samples leads one to conclude that either there simply were no oocysts in these environments or soil sampling, may not be a reliable method for evaluating a whole landscape and the probability of a small, native mammal ingesting an infective oocyst.

4.5 Recommendations for further research

In order to fill knowledge gaps, it is recommended that further work be undertaken to investigate the health of free-ranging southern brown bandicoot populations, including causes of death and the prevalence and effects of disease, particularly infection with *T. gondii*. This investigation would include further necropsy series on recovered cadavers followed by testing for the presence of *T. gondii* by qPCR as well as for other viral and microbiological pathogens. In addition, further experimental work, similar to Bettiol (2000) which experimentally infected eastern barred bandicoots with a known dose and strain of *T. gondii*, is recommended to study the pathogenesis of infection with local strains of *T. gondii* on individual southern brown bandicoots. While results obtained in experimental circumstances are difficult to compare with free-ranging populations, this would increase our knowledge about infection with *T. gondii* in this species and inform whether it is likely to be contributing to population decline. Longitudinal and cross-sectional field studies on free-ranging populations are recommended, to ascertain the effect of endemic infections with *T. gondii*, including morbidity, mortality, fecundity and demographic impacts. Ideally, these studies would encompass several years and different seasons, to allow for stochasticity of environmental conditions, larger sample sizes and individual identification. Such studies should be conducted in sites with a known higher environmental presence of *T. gondii*, where also cat density and seroprevalence studies have been performed (Fancourt and Jackson 2014; Fancourt *et al.* 2014).

Further studies are recommended to explore the feasibility of using eutherian species, especially mice and rabbits, as indicators for probability of infection with *T. gondii* of endangered and vulnerable species and to inform their conservation management. These would include studies to investigate whether free-ranging mice in a range of Australian conditions are becoming infected with *T. gondii* and to determine if vertical transmission of the parasite is occurring. Once these factors are known, studies are recommended across a range of seasons, geographical zones and a range of different levels of known *T. gondii* contaminated environments. The use of rabbits and mice as sentinels would be an invaluable tool for conservation management, including informing feral cat control programs and preparing Disease Risk Analyses for translocations. Black rats (*Rattus rattus*) and brown rats (*Rattus norvegicus*) are two other species that may be worthy of investigating as potential sentinel species for the presence of environmental *T. gondii* as has been shown in Cyprus (Psaroulaki *et al.* 2010).

The use of Whatman Filter Paper as a field method of blood collection for serology, both in native mammals and potential sentinel species, is worthy of further investigation. As mentioned in Chapter Three, this method of sampling would simplify blood collection, handling, storage and transportation.

4.6 Concluding remarks

This thesis has investigated the role that *T. gondii* may play in the health of the southern brown bandicoot in the northern hinterland of Western Port, Victoria and whether eutherian species, such as rabbits and mice can act as sentinel species to inform about probability of infection with *T. gondii* in small marsupials. The largest cause of death in a necropsy series of recovered, dead southern brown bandicoots was found to be motor vehicle trauma, followed by predation. There was no evidence of infection with *T. gondii* found in either necropsied or free-ranging animals. Environmental contamination with *T. gondii* was investigated in the same area. Field studies compared the prevalence of IgG antibodies to *T. gondii* in live trapped southern brown bandicoots with the prevalence of infection with *T. gondii* in two eutherian sentinel species and the prevalence of *T. gondii* oocyst DNA in soil samples. The study concluded that, in the

locations sampled and at the time of sampling, there was minimal evidence of environmental contamination with *T. gondii* and no evidence to suggest that the health of resident southern brown bandicoots was at risk through exposure to *T. gondii*. It also concluded that mice and rabbits may be useful sentinel species to inform about environmental contamination with *T. gondii* for conservation management of Australian native mammals. Further research is recommended to validate this in a range of different environments with varying cat densities and climatic conditions.

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