

Investigation of the risk of *Toxoplasma gondii* to the establishment of the 'extinct in the wild' eastern barred bandicoot (*Perameles gunnii*) on Phillip Island.



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## Abstract

The eastern barred bandicoot, *Perameles gunnii*, is currently considered extinct in the wild, and is extant only as intensively managed reintroduced populations. Phillip Island, in Westernport Bay, Victoria, has been proposed as a potential reintroduction site for this species. Feral cats, *Felis catus*, the definitive host for the coccidian parasite *Toxoplasma gondii*, are present on the island, and eastern barred bandicoots are known to be susceptible to toxoplasmosis. The aim of this study was to investigate the epidemiology of *T. gondii* on Phillip Island, and the potential risk to the establishment of eastern barred bandicoot populations.

The prevalence of *T. gondii* in the feral cat population on Phillip Island was determined using real time PCR (qPCR), and seroprevalence was determined using the modified agglutination test (MAT). A total of 161 feral cats was sampled between June 2016 and November 2017. Overall prevalence by qPCR was 79.5 % (95 % confidence interval [95% CI] 72.6 - 85.0), and overall seroprevalence was 91.8 % (95% CI 84.6 – 95.8). Additionally, the diagnostic sensitivity and specificity of both testing methods, and the true prevalence of *T. gondii* in feral cats on Phillip Island, were evaluated using Bayesian modelling. The sensitivity and specificity of the MAT in cats were estimated as 96.1 % (95 % credible interval [95% CrI] 91.5 – 98.8) and 82.0 % (95% CrI 65.1 – 93.3), respectively. The sensitivity and specificity of the qPCR method in cats were estimated as 90.1 % (95% CrI 83.5 – 95.6) and 96.2 % (95% CrI 82.2 – 99.8), respectively. The true prevalence of *T. gondii* infection in feral cats on Phillip Island was estimated as 90.2 % (95% CrI 83.1 – 95.2).

Environmental contamination with *T. gondii* oocysts was assessed. Soil was collected from two sites on the island, Summerland Peninsula and Cape Woolamai, in September and October 2017. A total of 412 soil samples, from 206 sites, was tested. Soil samples were processed to isolate any protozoan organisms present and qPCR was conducted to specifically detect *T. gondii* DNA. *Toxoplasma gondii* oocysts were not detected by qPCR in any of the soil samples collected.

Additionally, European rabbits, *Oryctolagus cuniculus*, were used as an indicator species to predict environmental exposure in eastern barred bandicoots. A total of 134 feral rabbits was sampled from August 2016 to November 2017. Overall prevalence of *T. gondii* in feral rabbits, as determined by qPCR on tissue, was 10.5 % (95% CI 6.3 - 16.8). *Toxoplasma gondii* infection was not identified in any rabbits collected from the proposed release site for eastern barred bandicoots, the Summerland Peninsula.

Sixty-seven eastern barred bandicoots were released onto the Summerland Peninsula in October and November 2017 as part of an assisted colonisation trial. Sixty-one of these bandicoots were tested for prior exposure to *T. gondii* using the MAT. No animals were seropositive for *T. gondii* prior to release on to Phillip Island, and the population could be concluded to be free from disease, at a design prevalence of 6 %. Bandicoots were trapped for blood sample collection in November 2017 after approximately three weeks of habitation on the island (n = 23), and again in February 2018 after approximately four months (n = 21). No bandicoots demonstrated seroconversion to *T. gondii* on the MAT in either November or February. In November, this was sufficient to conclude that the population was free from infection with *T. gondii*, at a design prevalence of 6 %. However, in February, the sample size was too small to make this conclusion. Mortality data on the bandicoots was limited, due to difficulties associated with placing radio-transmitters on this species. However, one deceased animal was recovered. This animal had no evidence of *T. gondii* infection based on qPCR on tissues.

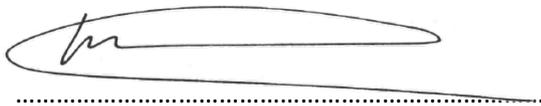
These findings suggest that while prevalence of *T. gondii* infection is very high in feral cats on Phillip Island, other factors, such as low cat density at the site, may translate to a low toxoplasmosis risk for eastern barred bandicoots released onto the Summerland Peninsula. Further studies are required to understand the factors contributing to the observed low level of environmental contamination with oocysts. Additionally, future studies should focus on increasing the sensitivity of mortality

surveillance in the eastern barred bandicoot population on the Summerland Peninsula. This will enable the collection of more robust mortality data, further elucidating the importance of toxoplasmosis in this population.

## Declaration by Author

I certify that:

- i. This thesis comprises only my original work towards the degree of Masters of Veterinary Science, except where due acknowledgment has been made in the text.
- ii. The thesis is fewer than 50,000 words in length, exclusive of tables, maps, bibliographies and appendices.



Katherine Jane Adriaanse

05 / 02 / 2019

Date

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# 1 Introduction

## 1.1 The eastern barred bandicoot (*Perameles gunnii*)

### 1.1.1 Species overview

The eastern barred bandicoot, *Perameles gunnii*, is an omnivorous marsupial that inhabits perennial tussock grasslands and grassy woodlands (Dufty, 1994). Its diet is largely insectivorous, consisting primarily of oligochaetes (earthworms); occasional plant material is also consumed. It is a small species, <1 kg in body weight, placing it within the 'critical weight range' of small mammals threatened with extinction in Australia (Johnson and Isaac, 2009). Historically, eastern barred bandicoots were widespread throughout south-western Victoria and a small adjacent area of south-eastern South Australia (Figure 1.1) (Seebeck et al., 1990). A distinct, unnamed subspecies is found in Tasmania. Dramatic population declines have occurred in the mainland subspecies since European colonisation, predominantly due to habitat loss as a result of agricultural clearing, predation by feral species, and other anthropogenic causes (Seebeck et al., 1990; Winnard and Coulson, 2008). By the 1980s, the mainland subspecies had contracted down to a small extant population located near Hamilton, in Western Victoria. This population was identified as critically small, and as a result, this subspecies has been the subject of a recovery program since 1989 (Winnard and Coulson, 2008). Despite recovery program efforts, the last wild population was declared extinct in 2008 (Winnard et al., 2013a).

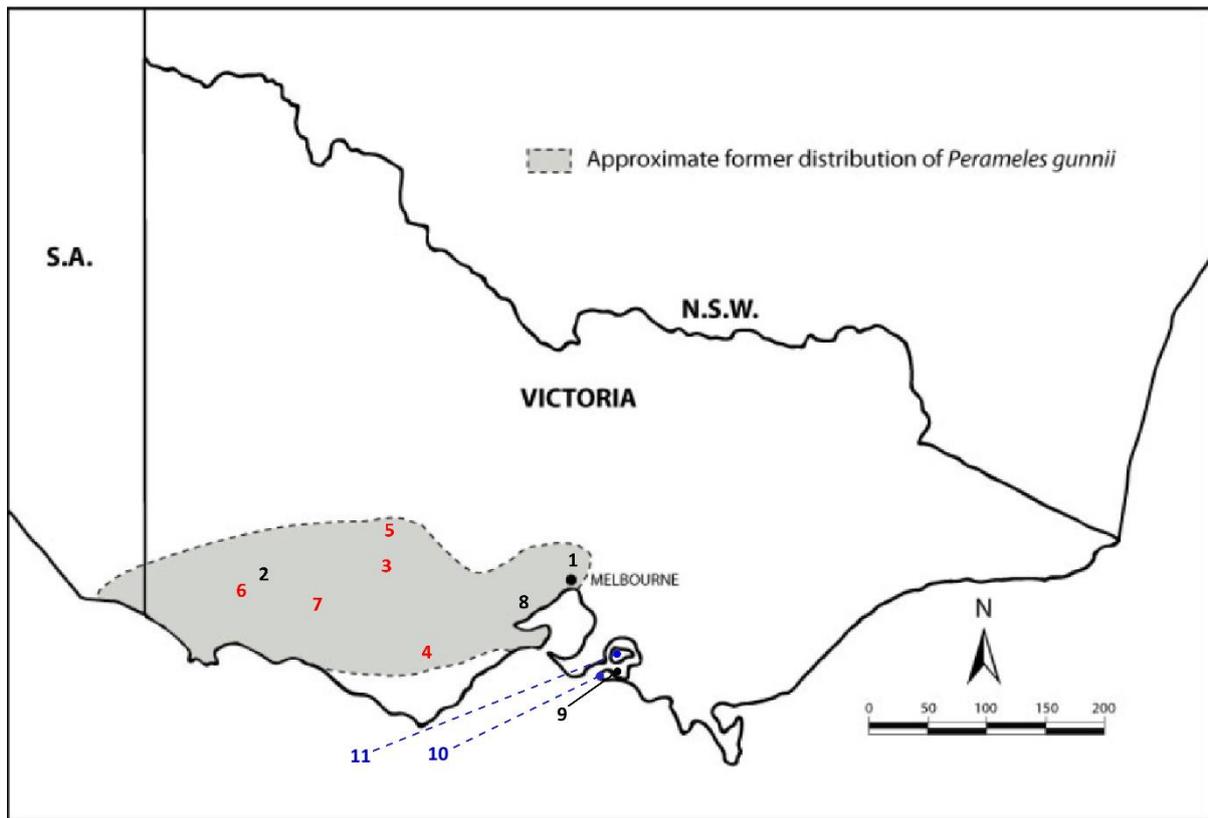


Figure 1.1: Former distribution of the eastern barred bandicoot, *Perameles gunnii*, mainland subspecies, in Australia and the locations of current, past, and proposed reintroduction sites. (Adapted from Winnard and Coulson, 2008)

Legend: Extant populations: 1- Woodlands Historic Park; 2 – Hamilton Community Parklands; 8 – Mt Rothwell; 9 – Churchill Island. Unsuccessful (past) reintroduction sites (red): 3– Mooramong; 4 – Floating Islands Nature Reserve; 5 – Lake Goldsmith Wildlife Reserve; 6 – Lanark; 7 – Cobra Killuc Wildlife Reserve. Proposed future reintroduction sites (blue): 10- Phillip Island; 11- French Island.

### 1.1.2 Species reintroduction efforts

The eastern barred bandicoot species recovery effort has consisted primarily of captive breeding and closely monitored reintroductions. A total of nine reintroduction sites, located throughout Victoria, have been trialled over the course of the recovery program (Figure 1.1) (Parrott et al., 2017; Winnard and Coulson, 2008). Four of these sites currently maintain extant populations: Hamilton Community Parklands, Woodlands Historic Park, Mt Rothwell Conservation and Research Centre, and Churchill Island. Factors contributing to the lack of success at the five failed reintroduction sites include: predation by foxes (*Vulpes vulpes*), habitat degradation by overpopulated grazing species (e.g. eastern grey kangaroos, *Macropus giganteus*, and European rabbits, *Oryctolagus cuniculus*),

drought, and disease (Winnard and Coulson, 2008). The Hamilton Community Parklands, Woodlands Historic Park, and Mt Rothwell sites are all enclosed in “predator-proof” fencing and have ongoing predator surveillance and control programs. Churchill Island, a 57-ha island in Westernport bay, Victoria, is geographically isolated from the Australian mainland, and is free from foxes and feral cats (*Felis catus*). This site therefore does not have the costs and practical difficulties associated with maintaining a predator-proof fence. Churchill Island is the most recent reintroduction site and is located outside of the species’ historic range. Twenty individuals were released onto Churchill Island between August and October 2015, and the population has since more than doubled (D. Sutherland, unpublished data). The total population of eastern barred bandicoots (captive and reintroduced) is managed as a ‘meta-population’, and is currently estimated at 1,200 individuals (Parrott et al., 2017).

### 1.1.3 French Island introduction trial

The recovery effort for this species is managed by a team consisting of members of different stakeholder groups and species experts: the Eastern Barred Bandicoot Recovery Team. This team aims to establish a self-sustaining population which totals a minimum of 2,500 individuals, although a recent population viability analysis has suggested a meta-population of  $\geq 3,000$  may be required (Hill et al., 2010; Lees et al., 2013). Population density estimates, based on data from historical free-ranging populations and reintroduction sites, range from 0.45 - 5.25 bandicoots/hectare with wide seasonal fluctuation (Dufty, 1991; Lees et al., 2013). Typical population densities are 0.7 - 1 bandicoots/hectare of suitable habitat (Lees et al., 2013). Thus, a minimum of 3,000 ha of suitable habitat is required to support such a population. Providing a predator-proof area of this size within the historic range of the eastern barred bandicoot is cost prohibitive (Hill et al., 2010).

Assisted colonisation is defined as “the intentional movement and release of an organism outside its indigenous range to avoid extinction of populations of the focal species” by the IUCN (IUCN/SSC,

2013). It is an important tool in species recovery programs when habitat cannot be secured within a species' historic range, and has already proved successful for eastern barred bandicoots on Churchill Island (Parrott et al., 2017). French Island, an 18,000 ha fox-free island in Westernport Bay, Victoria, has been identified as another potential site for assisted colonisation of the species, and has approximately 9,000 ha of suitable habitat (Figure 1.1) (Hill et al., 2010).

In 2012, an assisted colonisation trial was conducted on French Island (Groenewegen et al., 2017). Eighteen non-breeding bandicoots, fitted with intra-peritoneal radio transmitters, were released. Seven animals were known to survive longer than three months post-release. In a breeding population, this would be sufficient time for the first generation of offspring to reach independence (Groenewegen et al., 2017). Nine animals were confirmed to have died and two individuals were lost to follow up. Cause of death was determined in five of the mortality events. Two animals were predated by cats in the first week post-release. Two animals likely died of toxoplasmosis in the first month (Groenewegen et al., 2017). One of these animals had confirmed disseminated toxoplasmosis. The other animal was autolysed when recovered, however, a necrotic focus associated with protozoa assumed to be *T. gondii* was found in the pancreas (M. Lynch, unpublished data). Disseminated toxoplasmosis could not be confirmed due to the state of preservation of the carcass. A third animal was euthanased secondary to complications caused by the presence of the intra-peritoneal radio transmitter. This animal was found to have disseminated toxoplasmosis on histological examination of tissues collected at post mortem (Coetsee et al., 2016; Groenewegen et al., 2017). Blood samples were collected from the bandicoots three months after their release onto French Island and tested for serological evidence of *T. gondii* exposure using the modified agglutination test. One bandicoot tested positive but could not be located again due to transmitter failure (Groenewegen et al., 2017). French Island was known to have a feral cat population at the time of the release; however, planned feral cat control could not be carried out prior due to unseasonably wet conditions (Groenewegen et al., 2017). There are no available estimates for cat

density at the time of release but it is thought to have been very high as the cat control program on French Island was in its early stages (the program began in 2010), and there were several 'cat factories' (sheds where feral cats were known to breed in large numbers) located close to the release site (A. Coetsee, personal communication). In addition, the wet conditions likely contributed to the survival of *T. gondii* oocysts and thus increased the risk of exposure for bandicoots.

Accordingly, it was noted that all bandicoots shown to have exposure to *T. gondii* on French Island were inhabiting low-lying, sometimes water-logged areas (Groenewegen et al., 2017). Despite these deaths, the post-release survival targets ( $\geq 50\%$  survival at 1 month, and  $\geq 10\%$  survival at 6 months) of this trial were still met. At the end of the trial, any surviving bandicoots that could be trapped were returned to Melbourne Zoo. The study concluded that assisted colonisation of fox-free islands remains the best chance for the wild recovery of this species (Groenewegen et al., 2017). However, the study also identified toxoplasmosis as a significant threat to the successful establishment of eastern barred bandicoots at release sites and highlighted the need to consider feral cat control at these locations.

#### 1.1.4 Phillip Island as a potential introduction site

Phillip Island, a 10,000 ha island located in Westernport Bay, Victoria, has also been identified as a possible release site for eastern barred bandicoots, and is expected to provide  $> 8,000$  ha of suitable habitat (Figure 1.1) (Coetsee, 2016; Parrott et al., 2017). Similar to French and Churchill Islands, Phillip Island is outside of the historic range of the species. However, the apparent success of the introduction on Churchill Island suggests that eastern barred bandicoots can establish on sites outside this range. Phillip Island maintains a strict fox control program and has recently been declared fox-free (D. Sutherland, personal communication). However, the island does have a significant feral cat population. Therefore, as for French Island, toxoplasmosis and direct predation are important risk factors to consider in regard to the establishment of a new bandicoot population (Groenewegen et al., 2017). In contrast to French Island at the time of the 2012 trial, Phillip Island

has a well-established ongoing feral cat control program, managed by Phillip Island Nature Parks. This is likely to reduce environmental contamination with *T. gondii* oocysts and reduce the risk of predation.

## 1.2 *Toxoplasma gondii*

### 1.2.1 Taxonomy

*Toxoplasma gondii* is a protozoan parasite, belonging to the Phylum Apicomplexa, Subclass Coccidiasina (Dubey, 2010). *Toxoplasma gondii* is the only recognized species of the genus *Toxoplasma* and has a cosmopolitan distribution. While felids are the only definitive host, *T. gondii* can use any 'warm-blooded' animal (i.e. mammals and birds) as an intermediate host. Due to its wide distribution, host flexibility, and ability to cause disease in animals and humans, *T. gondii* has been studied widely.

### 1.2.2 Lifecycle

*Toxoplasma gondii* has a complex, heteroxenous lifecycle (Figure 1.2). There are three infective stages: oocysts, tachyzoites, and bradyzoites (Dubey, 2010). Oocysts are the environmental stage of *T. gondii*, whereas tachyzoites and bradyzoites are found within host tissues.

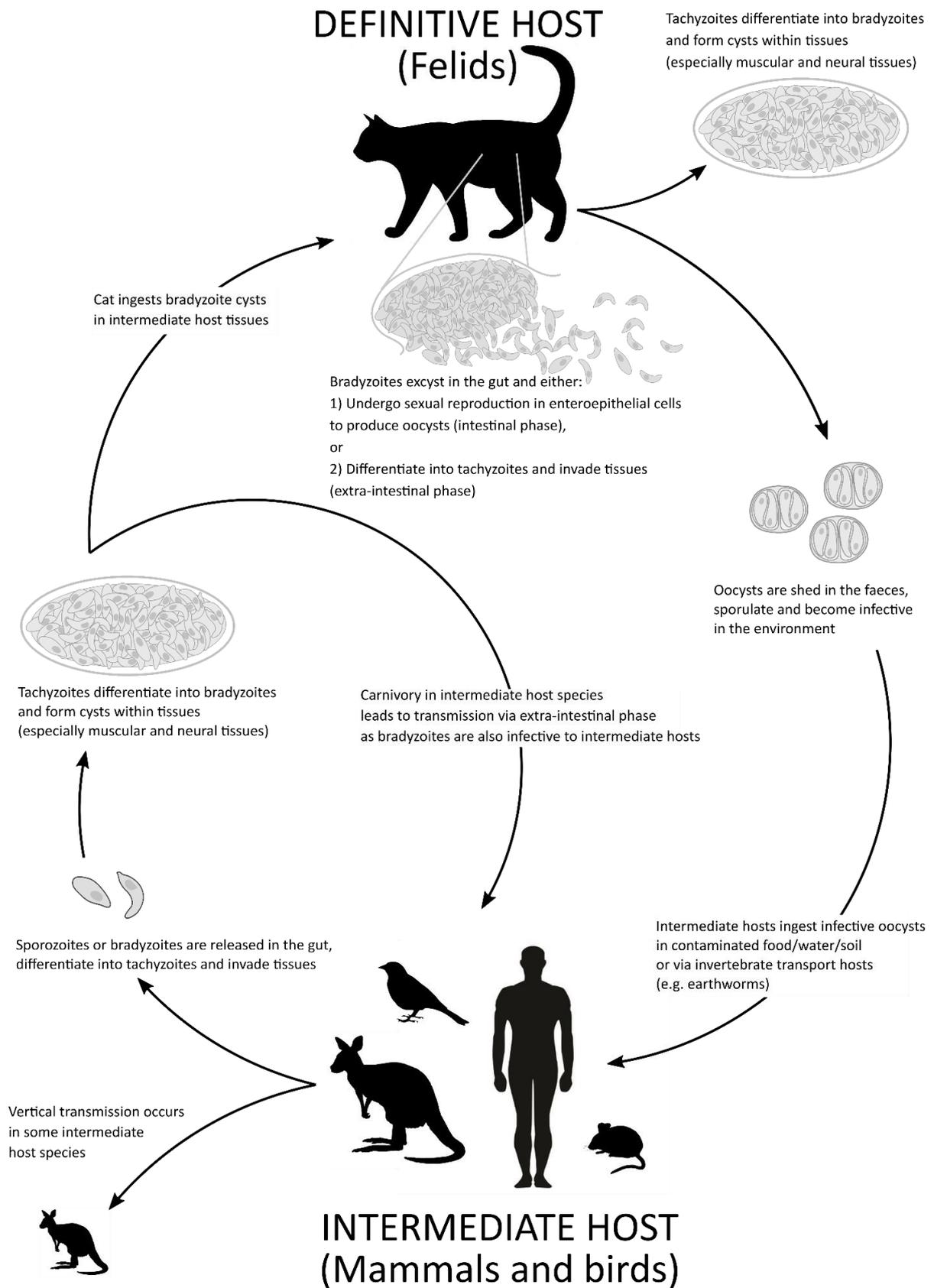


Figure 1.2: Lifecycle of the protozoan parasite *Toxoplasma gondii*.

Oocysts are formed by sexual reproduction of *T. gondii*, which occurs only in the intestine of felids, the definitive hosts for the parasite (Dubey, 2010). Oocysts are shed in the faeces; the shedding period lasts 1-2 weeks, and millions of oocysts may be shed from a single individual (Dubey, 2001). Cats are often assumed to shed oocysts only once in their lifetime (Davis and Dubey, 1995). However, under experimental conditions, re-exposure to *T. gondii* after an extended period (> 6 years) (Dubey, 1995), immunosuppression (Dubey and Frenkel, 1974), and co-infection with *Cystoisospora felis* (Chessum, 1972; Dubey, 1976), have all been shown to lead to repeat shedding. In addition, Dubey and Frenkel (1974) showed that cats infected as kittens shed oocysts on re-challenge, compared to those infected as adults, which did not. When cats did have repeat oocyst shedding, numbers were lower than for the initial infection (Dubey and Frenkel, 1974). More recently, Zulpo et al. (2018) also demonstrated re-shedding of oocysts after challenge with heterologous strains. They found that immunity to oocyst re-shedding waned over time, with 90% of cats protected against re-shedding at 12 months compared to 25% at 24 months. All cats seroconverted after initial challenge; antibody levels were maintained throughout the study and were not associated with protection against re-shedding of oocysts (Zulpo et al., 2018). Similarly to Dubey and Frenkel (1974), Zulpo et al. (2018) also found that cats shed lower numbers of oocysts in subsequent infections. However, they postulated that since adult cats produce a greater volume of faeces than kittens, overall environmental contamination due to re-infected adult cats is only 30% lower than that caused by infected kittens. It is difficult to directly extrapolate these experimental studies to naturally-infected cats; however, it is reasonable to assume that re-shedding of oocysts after initial infection is possible. Nevertheless, if one assumes free-roaming cats are exposed soon after weaning (when they begin to consume potentially infected animal tissues), kittens and young cats are likely to be the most important source of environmental contamination. It is estimated that approximately 1 % of a cat population will be shedding oocysts at any one time (Dubey, 2010). Once in the environment, oocysts take 1-5 days to sporulate and become infective (Dubey, 1998). An infective oocyst consists of two sporocysts, each containing four sporozoites (Dubey, 1998). Oocysts

are extremely robust, and can survive in soil for up to 18 months in ideal conditions (Frenkel et al., 1975). They are susceptible to desiccation and may be destroyed by extremely hot temperatures (Dubey, 1998; Yilmaz and Hopkins, 1972). Intermediate hosts can be infected by the ingestion of oocysts in contaminated feed, water, or soil. After ingestion, sporozoites excyst in the gut of the intermediate host and invade many different cell types (Dubey, 2010). From there, they begin to multiply and eventually form bradyzoite tissue cysts, if the host survives the acute infection (Dubey, 2010). In mice, bradyzoites and tissue cysts have formed by 6 days post-infection (Dubey et al., 1997). Felids may also be infected via ingestion of oocysts; however, this pathway is much less efficient for infecting felid species than ingestion of tissue cysts (Dubey, 2006).

Bradyzoites and tachyzoites are formed by endodyogeny, a type of asexual reproduction common to apicomplexan protozoans (Dubey, 2010). Tachyzoites are the rapidly dividing phase of *T. gondii* and are found within host tissues (Dubey, 1998). Bradyzoites are similar in structure to tachyzoites, but divide slowly in tissues and are responsible for the development of tissue cysts that persist within the host (Dubey, 1998). Tissue tropism differs between hosts, but cysts are typically found in the greatest numbers in muscular and neural tissue (Di Cristina et al., 2008; Dubey et al., 2016). When a felid preys on an infected intermediate host, they are infected via ingestion of *T. gondii* bradyzoites within tissue cysts. Tachyzoites are also infectious if ingested, although this pathway is probably less important in nature. After ingestion of a tissue cyst by a felid, bradyzoites excyst and enter cells within the gut to undergo sexual reproduction (intestinal phase) and produce oocysts, which are excreted, completing the lifecycle (Dubey, 2010). In addition to sexual reproduction, asexual reproduction will also occur, and infected felids also carry *T. gondii* bradyzoite cysts within their tissues (extra-intestinal phase) (Dubey, 2010). Carnivorous and omnivorous intermediate hosts, including humans, can also be infected by ingestion of these tissue cysts. This carnivorous cycle allows *T. gondii* to persist in ecosystems, even if oocysts are unable to remain infective under harsh environmental conditions. In fact, *T. gondii* can even persist in the absence of felids, where

migratory intermediate hosts can lead to infection in non-felid carnivores (Prestrud et al., 2007).

Tissue cysts can be inactivated by cooking and, in humans, increased risk of exposure to toxoplasmosis is associated with eating rare or undercooked meat (Jones et al., 2009).

*Toxoplasma gondii* may also be transmitted vertically, from mother to foetus. In humans, this is considered only to occur if an immuno-naïve mother is acutely infected during pregnancy, or due to recrudescence if the mother is immunocompromised (e.g. Acquired Immunodeficiency Syndrome, AIDS), as tachyzoites are capable of crossing the placenta (Dubey, 2010). However, in mice, *T. gondii* may be transmitted through several generations without repeated exposure to the parasite (Owen and Trees, 1998). Vertical transmission in chronically infected dams has also been demonstrated in rabbits (Uhlíková and Hübner, 1973), sheep (Williams et al., 2005), and Australian marsupials (Parameswaran et al., 2009). It is suspected that immunosuppression associated with pregnancy in these species leads to recrudescence of latent toxoplasmosis, therefore resulting in congenital infection of the foetus.

### 1.2.3 Population structure of *Toxoplasma gondii*

Despite the presence of a sexual reproductive phase within its lifecycle, *T. gondii* typically exhibits a highly clonal population structure (Sibley and Ajioka, 2008). This is dominated by three main lineages in Europe and North America, type I, II, and III (Howe and Sibley, 1995). Genotype appears to be associated with virulence, for example, Howe and Sibley (1995) found that most human toxoplasmosis cases were associated with type II strains, whereas subsequent studies suggested that human ocular toxoplasmosis was more strongly associated with type I strains (Grigg et al., 2001). Recent studies have identified a fourth clonal lineage in North America, type 12, that is highly prevalent in wildlife (Khan et al., 2011). In contrast, the population structure of *T. gondii* is markedly different in South America, and shows much greater genetic diversity (Ajzenberg et al., 2004). While asexual propagation appears to be the main method of reproduction in North America and Europe,

South American strains show more evidence of recombination, suggesting that sexual reproduction occurs more frequently in these populations (Sibley and Ajioka, 2008; Su et al., 2012). Recent studies have indicated a South American origin for *T. gondii*, and it has been proposed that an ancestral form of the parasite entered South America with the Felidae where it diversified and expanded along with its host species (Bertranpetit et al., 2017). This theory explains the genetic diversity of strains present in South America. The diversity of host species present in sylvatic cycles in this region likely serves to maintain this genetic diversity (Ajzenberg et al., 2004). Furthermore, it has been suggested that the clonal nature of *T. gondii* in other parts of the world is a consequence of the more recent expansion of the domestic cat, *F. catus*, which may have favoured a small number of well-adapted genotypes (Müller and Howard, 2016).

Studies on the population genetics of *T. gondii* in Australia are limited. Type II strains have been identified in clinically affected and latently infected domestic cats (Brennan et al., 2016). In addition, type II strains have been identified in free-ranging wildlife with clinical toxoplasmosis, including common wombats (*Vombatus ursinus*) and a New Zealand fur seal (*Arctocephalus forsteri*) (Donahoe et al., 2014; Donahoe et al., 2015). Atypical strains have also been identified in Australia, in wildlife and in domestic species (Parameswaran et al., 2010). Pan et al. (2012) found that individual free-ranging macropods were simultaneously infected with multiple, genetically diverse *T. gondii* strains. It is often assumed that multiple strain infections, and therefore recombination through sexual reproduction, occur infrequently in the cat, contributing to the clonal nature of *T. gondii* populations (Sibley and Ajioka, 2008). However, these findings provide a putative mechanism for multiple strain infections in feline hosts through predation (Pan et al., 2012). This suggests that there is the potential for frequent genetic exchange through sexual reproduction and recombination, leading to a genetically diverse *T. gondii* population structure in Australia. It should be noted that the Pan et al. (2012) study examined macropod species (red kangaroos, *Macropus rufus*; western grey kangaroos, *M. fuliginosus*; and wallaroos, *M. robustus*) which are too large to be predated on by feral cats,

although cats may scavenge from carcasses. It is not known whether multiple strain infections are also seen in smaller marsupial species. Further studies are required to fully understand the population structure of *T. gondii* in the Australian environment.

#### 1.2.4 *Toxoplasma gondii* and clinical disease

Toxoplasmosis is the disease caused by infection with *T. gondii*. In general, immunocompetent hosts infected with the parasite experience no disease, or very mild clinical signs (Dubey, 2010). In humans, the most common symptoms of mild cases are cervical lymphadenopathy, malaise, and fever; many cases probably go undiagnosed (Weiss and Dubey, 2009). In immunocompromised individuals, or congenitally infected infants, toxoplasmosis can cause severe disease involving many organ systems. This includes chorioretinitis, myositis, myocarditis, pneumonitis, hepatitis, and encephalitis (Weiss and Dubey, 2009).

In addition to representing a risk to public health, *T. gondii* infections in domestic livestock may also cause significant economic losses. In particular, toxoplasmosis is an important cause of embryonic loss and abortion in sheep. In New Zealand, between 1973 and 1989, *T. gondii* was responsible for 7 – 32 % of all outbreaks of ovine abortion investigated in a given year (Gumbrell, 1990).

Comparatively, toxoplasmosis was estimated to be responsible for 1 – 2 % of all neonatal losses in sheep per year in the United Kingdom (Blewett and Trees, 1987). In Australia, there are limited studies addressing the proportion of ovine abortions that can be attributed to toxoplasmosis and the resultant economic losses to the sheep industry. Additionally, under-reporting is likely, as infected dams are clinically well and therefore detailed investigations may not be carried out, particularly in cases of early embryonic loss. Studies have estimated flock seroprevalence of up to 41 %, and up to 25 % seroprevalence in individual sheep in Australia (O'Donoghue et al., 1987; Plant et al., 1982).

*Toxoplasma gondii* can also cause clinical disease in its definitive hosts, cats and other members of the family Felidae. As is seen in other host species, immunocompetent adult felids typically experience subclinical disease (Dubey, 2010). Severe systemic disease can occur in neonates and immunosuppressed individuals, such as animals treated with high doses of corticosteroids (Dubey, 1996a), or individuals infected with Feline immunodeficiency virus (Davidson et al., 1993). Sporadic cases of disseminated toxoplasmosis in immunocompetent cats are also reported (Nagel et al., 2013; Spycher et al., 2011). It has been suggested that these cases may occur due to unique host or strain characteristics, although the specific factors involved have not been elucidated (Spycher et al., 2011). Interestingly, severe disease is also seen in some species of non-domestic felid, such as Pallas's cat (*Otocolobus manul*); the reasons for this are not yet known (Basso et al., 2005). Clinical signs of toxoplasmosis in cats are non-specific and varied. They include fever, anorexia, lethargy, vomiting and diarrhoea, respiratory signs (e.g. tachypnoea and dyspnoea), and neurological abnormalities (e.g. blindness, ataxia, circling, etc.) (Dubey, 2010).

#### 1.2.5 Significance of toxoplasmosis in Australian marsupials

Toxoplasmosis has been well-documented as a cause of fatal disease in Australian marsupials. These reports are typically from captive populations and include both sporadic individual cases and outbreaks. For example, in 1984, at the Knoxville Zoo, USA, 13 marsupials became acutely ill and died of toxoplasmosis over a 10 day period (Patton et al., 1986). Species affected included eastern grey kangaroos (*M. giganteus*), red kangaroos (*M. rufus*), Tammar wallabies (*M. eugenii*), and long-nosed potoroos (*Potorous tridactylus*) (Patton et al., 1986). A wide range of marsupial species have been reported to be affected by toxoplasmosis in the literature, including macropods, koalas (*Phascolarctos cinereus*), eastern barred bandicoots (*P. gunnii*), sugar gliders (*Petaurus breviceps*), and common wombats (*V. ursinus*) (Table 1.1). Toxoplasmosis has also been reported in the Australian monotremes, the short-beaked echidna (*Tachyglossus aculeatus*) and platypus (*Ornithorynchus anatinus*) (McColl, 1983; McOrist and Smales, 1986). In Australia, there are no

native felid species, and the only known primary host for *T. gondii* is the introduced cat (*F. catus*), either domestic or feral. Cats were introduced to Australia during British colonisation in the late 1700s, and feral cats are now widespread across the continent (Doherty et al., 2016). Australian marsupials are often considered to be among the most susceptible species to toxoplasmosis, and it is thought that this is due to lack of exposure to felids and their parasites throughout the majority of their evolutionary history (Innes, 1997).

The clinical signs of toxoplasmosis in marsupials, outlined in Table 1.1, are varied. They include: respiratory signs such as dyspnoea, tachypnoea, and coughing (Canfield et al., 1990; Patton et al., 1986); neurological signs such as blindness, circling, ataxia, and nystagmus (Adkesson et al., 2007; Donahoe et al., 2015); gastrointestinal signs such as diarrhoea (in macropods) (Miller et al., 1992); and lameness, secondary to myositis (Portas, 2010). Toxoplasmosis may also cause subtle behavioural changes, such as increased diurnal activity in crepuscular or nocturnal species (Bettioli et al., 2000a; Portas, 2010). Uncharacteristic docility is a feature of toxoplasmosis commonly reported in eastern barred bandicoots (Lynch, 2008). Additionally, cases may present with non-specific signs such as depression, anorexia, and weight loss, or simply as sudden death with no premonitory signs (Canfield et al., 1990; Dubey et al., 1988).

Table 1.1: Australian marsupial species reported to have clinical toxoplasmosis, and clinical signs associated with disease.

Species affected	Clinical signs	Captive or Free-ranging?	Reference
<b>Common wombat</b> ( <i>Vombatus ursinus</i> )	Ataxia, blindness, circling, head tilt, weakness	Free-ranging	(Donahoe et al., 2015)
<b>Eastern barred bandicoot</b> ( <i>Perameles gunnii</i> )	Ataxia, blindness, abnormal daytime activity, death	Captive Free-ranging	(Bettiol et al., 2000a) (Lenghaus et al., 1990; Obendorf and Munday, 1990)
<b>Eastern grey kangaroo</b> ( <i>Macropus giganteus</i> )	Anorexia, weakness, blindness, respiratory distress, convulsions, death	Captive	(de la Cruz-Hernandez et al., 2012; Patton et al., 1986)
<b>Koala</b> ( <i>Phascolarctos cinereus</i> )	Respiratory disease, weight loss, death  Sudden death	Captive  Captive	(Dubey et al., 1991)  (Hartley et al., 1990)
<b>Long-nosed potoroo</b> ( <i>Potorous tridactylus</i> )	Lethargy, respiratory distress, death	Captive	(Patton et al., 1986)
<b>Red kangaroo</b> ( <i>Macropus rufus</i> )	Lethargy, respiratory distress, death	Captive	(Patton et al., 1986)
<b>Red-necked wallaby</b> ( <i>Macropus rufogriseus</i> )	Depression, unresponsiveness, ataxia, weight loss, diarrhoea, death  Blindness, ataxia, death	Captive  Free-ranging	(Adkesson et al., 2007; Basso et al., 2007; Bermúdez et al., 2009; Miller et al., 1992)  (Obendorf and Munday, 1983)
<b>Sugar glider</b> ( <i>Petaurus breviceps</i> )	Inappetence, weakness, seizures, sudden death	Captive	(Barrows, 2006)
<b>Tammar wallaby</b> ( <i>Macropus eugenii</i> )	Depression, ataxia, respiratory distress, sudden death	Captive	(Lynch et al., 1993; Patton et al., 1986)
<b>Tasmanian pademelon</b> ( <i>Thylogale billardierii</i> )	Blindness, ataxia, death	Free-ranging	(Obendorf and Munday, 1983)
<b>Wallaroo</b> ( <i>Macropus robustus</i> )	Sudden death (adults), diarrhoea (joey), weight loss (joey)	Captive	(Boorman et al., 1977)
<b>Western grey kangaroo</b> ( <i>Macropus fuliginosus</i> )	Sudden death in joeys	Captive	(Dubey et al., 1988)

In contrast with captive animals, clinical toxoplasmosis has been described in only a limited number of free-ranging marsupial populations (Table 1.1). Animals in captivity may be under a number of stressors, including suboptimal nutrition, overcrowding, and other husbandry factors, that could predispose them to toxoplasmosis. These issues can be subtle, and therefore it can be difficult to tease out the importance of *T. gondii* as a primary disease-causing agent in marsupials. In addition, these confounding factors mean that it is not sensible to simply extrapolate from captive populations and consider toxoplasmosis a significant disease of all wild marsupial populations. Toxoplasmosis is often implicated as a contributing factor in small mammal declines in Australia (Hollings et al., 2013; Thompson et al., 2010). However, data quality is variable for historic extinction events and evidence is often circumstantial; therefore, the role of *T. gondii* is debated (Hillman et al., 2016). As can be seen from Table 1.1, outbreaks of clinical disease have been observed in free-ranging populations, suggesting the disease may be significant. Conversely, many studies have also identified serological evidence of *T. gondii* exposure in several species without evidence of clinical disease. These species include, but are not limited to, the Tasmanian pademelon (*Thylogale billardierii*), red-necked wallaby (*M. rufogriseus*) and common wombat (*V. ursinus*) (Hartley and English, 2005; Hollings et al., 2013; Parameswaran, 2008). Interestingly, outbreaks of toxoplasmosis have also been reported in free-ranging populations of these three species (Table 1.1) (Donahoe et al., 2015; Obendorf and Munday, 1983). It is likely that other host, environmental, and agent factors contribute to outbreaks of disease. For example, in mice, type I strains of *T. gondii* are highly virulent, resulting in 100 % mortality regardless of infectious dose (Howe et al., 1996). Type II and III strains are comparatively less virulent, and chronic infections are established more easily from these strains (Howe et al., 1996). Infectious dose and host species have also been proven to be important in the development of clinical disease in experimental studies. Dubey (1996b) showed that all mice fed 100 or more oocysts of the VEG strain (a type III strain) died. In contrast, rats became ill with diarrhoea and depression when infected with up to  $10^5$  -  $10^6$  oocysts of the same strain, and deaths were only observed in those infected with  $10^6$  oocysts (Dubey, 1996b). The impact of these factors

on marsupial species is largely unknown, and the epidemiology of *T. gondii* in free-ranging populations is poorly understood.

#### 1.2.6 Significance of toxoplasmosis in eastern barred bandicoots

Toxoplasmosis is known to affect eastern barred bandicoots, and can cause incoordination, blindness, abnormal daytime activity, and death (Bettioli et al., 2000a; Lenghaus et al., 1990). Bandicoots are likely to be exposed to *T. gondii* by ingestion of oocyst contaminated soil, or via consumption of invertebrate prey (e.g. earthworms) that have *T. gondii* oocysts within their gut as a result of coprophagia. Fatal toxoplasmosis has been demonstrated in this species by feeding of experimentally contaminated earthworms (Bettioli et al., 2000b).

In 1984, an outbreak of neurological disease in a free-ranging Tasmanian population of eastern barred bandicoots was attributed to toxoplasmosis (Obendorf and Munday, 1990). Histological examination of deceased animals identified protozoan tissue cysts consistent with *T. gondii*; however, molecular techniques to further characterize these protozoa were not used (Obendorf and Munday, 1990). The lack of further molecular investigation was likely due to the limited availability of these techniques at the time of the outbreak. A study investigating the cause of death in free-ranging eastern barred bandicoots, from the now extinct mainland population near Hamilton, identified toxoplasmosis as the primary cause of death in three out of 63 cases submitted for histopathology (Lenghaus et al., 1990). Toxoplasmosis was considered a contributing factor to death in seven other cases. The primary cause of death in these cases was attributed to vehicular trauma (5 cases), predation (1 case), and trap death (1 case). Thus, overall, toxoplasmosis was thought to have caused or contributed to death in just under 16 % of cases.

Toxoplasma seropositive eastern barred bandicoots have been identified in both captive and free-ranging populations, indicating that individuals are able to survive initial infection and mount an

immune response. In a survey of 57 captive animals, 9 % were identified as seropositive (Miller et al., 2000). A similar proportion, 6.7 % (n = 150), of wild eastern barred bandicoots from Tasmania were identified as seropositive (Obendorf et al., 1996). Both studies used the modified agglutination test and classified a titre of  $\geq 1/64$  as positive. Five out of the 10 seropositive free-ranging individuals were never trapped again (Obendorf et al., 1996). One seropositive animal was found dead in the trap at a subsequent trapping session, and had extensive lesions associated with *T. gondii* infection on histological examination of tissues. Another was found to be weak and docile in the trap and when released displayed marked ataxia; this animal was never trapped again. The remaining three seropositive individuals were trapped twice, three months apart, and then not trapped again. This suggests that free-ranging eastern barred bandicoots may not survive long term after infection with *T. gondii*. Comparatively, 68 % of seronegative individuals were re-trapped during subsequent trapping periods (Obendorf et al., 1996). There are many factors which are likely to affect the outcome of infection, including parasite factors (e.g. infectious dose and genetic strain), and host factors (e.g. nutritional stress, age, and immune competence). In addition, behavioural changes caused by *T. gondii*, such as increased diurnal activity, may be insignificant in captive populations but may increase the likelihood of other causes of mortality (e.g. predation, motor vehicle trauma) in free-ranging animals.

### 1.2.7 Treatment of toxoplasmosis in Australian marsupials

Although various treatment regimens have been proposed for toxoplasmosis in Australian marsupials, treatment is frequently unrewarding (Lynch, 2008). A combination of sulphonamide and trimethoprim 25 mg/kg orally q24 h, pyrimethamine 0.5 mg/kg orally q24 h, and folic acid 1 mg/kg orally q24 h, or clindamycin 10-15 mg/kg q12 h for 4 weeks, have both been suggested (Johnson and Hemsley, 2008; Lynch, 2008). Dubey reported successful treatment of clinical toxoplasmosis in wallabies using atovaquone 100 mg/kg orally q24 h for extended periods, although *T. gondii* was not eliminated from the tissues of these animals (Dubey and Crutchley, 2008). Attempts to vaccinate

marsupials against *T. gondii* have been largely unsuccessful. Reddacliff et al. (1993) found that vaccination of Tammar wallabies with *Hammondia hammondi* may have conferred some protection upon challenge with *T. gondii*, but did not prevent infection. A commercial live vaccine for ovine toxoplasmosis (*T. gondii* S48 strain) was trialled in Tammar wallabies; however, four of six vaccinated animals died or developed severe clinical toxoplasmosis (Lynch et al., 1993). While further studies may allow the development of a safe and efficacious *T. gondii* vaccine for captive marsupials, it is unlikely that such a vaccine will be useful for free-ranging populations on a large scale.

#### 1.2.8 Detecting *Toxoplasma gondii* in mammalian hosts

Detection of the presence of, or exposure to, *T. gondii* in mammalian hosts can be achieved in several different ways. These techniques are used to diagnose toxoplasmosis and understand its role in outbreaks of clinical disease. Prevalence of *T. gondii* in hosts has also been used as a proxy for environmental contamination, when methods for direct environmental detection have been unavailable (Afonso et al., 2010). Serological techniques are the most commonly employed test type for ante mortem diagnosis of toxoplasmosis. There are several different techniques, with varied sensitivity and specificity, and no gold standard test exists for screening across the range of potential *T. gondii* host species. Serological tests include the Sabin–Feldman dye test, indirect hemagglutination assay (IHA), indirect fluorescent antibody assay (IFA), latex agglutination test (LAT), enzyme-linked immunosorbent assay (ELISA), immunosorbent agglutination assay test (IAAT), and the direct or modified agglutination test (DAT and MAT, respectively) (Hill and Dubey, 2002). Sensitivity and specificity are often dependent on the species in which the test is used, and tests have only been validated for a limited number of species. Tests may detect either IgG antibodies, IgM, or both. The MAT detects IgG antibodies directed against *T. gondii* (Dubey and Desmonts, 1987). The MAT does not require any specialist equipment, and, in particular, does not require the development of species-specific antibodies (in contrast to ELISA and IFA). For these reasons it has been used in the greatest number of different species (Robert-Gangneux and Dardé, 2012).

Regardless of the sensitivity and specificity, none of the tests can definitively prove the presence of acute toxoplasmosis in an animal. As with serological tests for many infectious diseases, paired sera demonstrating rising titres, temporally associated with clinical disease, are strongly indicative of toxoplasmosis. Detection of antibodies to *T. gondii* does confirm exposure to the parasite, and in the case of the definitive host, the cat, individuals have usually ceased shedding oocysts by the time of seroconversion (Dubey, 2010).

Oocysts may be detected in faeces of felids that are currently shedding. However, as only 1 % of a cat population is expected to be shedding at any one time, faecal analysis is not considered useful for epidemiological studies (Dubey, 2010). The sensitivity of microscopy is poor – the threshold of detection is approximately 1,000 oocysts/gram of faeces (Dubey, 2010). Additionally, related species, such as *H. hammondi* may be falsely identified as *T. gondii*. In a European study, *T. gondii*-like oocysts were detected in 48 of 24,106 cat faecal samples; however, 22 were later identified as *H. hammondi* by PCR (Schaes et al., 2008b). Molecular methods can increase the sensitivity of detection in faeces. *Toxoplasma gondii* DNA was identified in 11 of 122 feline faecal samples where oocysts could not be detected by microscopy (Salant et al., 2007). However, PCR detects DNA only, and thus the presence of viable oocysts cannot be confirmed using this technique.

Conventional PCR is a highly sensitive and specific test for the presence of *T. gondii* DNA in host tissues (Burg et al., 1989). A number of gene targets have been reported. The B1 gene is commonly targeted, as it is repeated up to 35 times within the *T. gondii* genome, and therefore provides good test sensitivity (Burg et al., 1989). Conventional PCR may also be performed as a nested or hemi-nested PCR which further increases sensitivity. More recently, a 529 bp highly repetitive element has been identified as a target. This sequence is present 200-300 times in the *T. gondii* genome (Homan et al., 2000). The 529 bp repetitive element is claimed to be 10-100 times more sensitive than B1 (Dubey, 2010). Real-time quantitative PCR (qPCR) assays have also been developed to both gene

targets. The advantages of qPCR are the ability to rapidly screen large numbers of samples, and the capacity to quantify the amount of *T. gondii* DNA present in a sample, which is useful for both clinical and epidemiological applications. In addition, TaqMan probe-based qPCR significantly adds to assay specificity based on the use of highly species-specific hybridization probes. Loop mediated isothermal amplification (LAMP) has also been used for the detection of *T. gondii* DNA in tissue samples (Zhang et al., 2009). Although LAMP has slightly reduced sensitivity compared to qPCR, it shows promise as a sensitive, specific, and rapid test which may be suitable for field applications (Lin et al., 2012). Despite the high tissue specificity and sensitivity of DNA-based detection methods, overall host-level sensitivity may be lower than serological methods (Bacci et al., 2015; Garcia et al., 2006a; Hill et al., 2006). This is primarily due to the focal and random distribution of *T. gondii* organisms within host tissues, particularly in asymptomatic chronic infections, which may lead to a lack of parasites in the portion of tissue which is sampled (Hill et al., 2006). Additionally, tissue tropism varies between species and individuals, and therefore false negatives may occur if the wrong tissue types are sampled (Dubey, 1997). These issues can be counteracted in part by using large tissue samples, ensuring tissues are well homogenised prior to DNA extraction, and selecting several different tissues from each individual, based on known tissue tropism for that species. Other methods used to increase sensitivity include *T. gondii* sequence-specific magnetic capture DNA isolation (Opsteegh et al., 2010). This technique decreases the amount of background host DNA obtained during the DNA isolation step and can be combined with qPCR (known as MC-PCR) to increase the sensitivity of detection, particularly in large volume tissue samples (Opsteegh et al., 2010).

*Toxoplasma gondii* tachyzoites and bradyzoites may also be detected in tissue by direct smears and histopathology. This method is insensitive and *T. gondii* may be missed in low level infections. Well preserved tachyzoites may be easily identified; however, if degradation has occurred during the disease process or sample preparation, immunohistochemical stains are required in order to

definitively differentiate *T. gondii* from closely related protozoan species (Dubey, 2010).

Nevertheless, histological examination provides evidence of tissue pathology, which can differentiate between subclinical and clinical forms of toxoplasmosis.

#### 1.2.9 *Toxoplasma gondii* in feral cats in Australia

Several studies have surveyed feral cat populations across Australia for antibodies to *T. gondii* (Table 1.2). Seroprevalence ranges from 0 % to up to 96 % in some locations (Adams, 2003; Adams et al., 2008; Jakob-Hoff and Dunsmore, 1983). Direct comparison of reported prevalence between locations is difficult, due to the use of different serological tests across the studies.

There is limited information in the literature regarding the prevalence of *T. gondii* in feral cats in Australia as detected by PCR. Adams (2003) detected *T. gondii* in 4.9 % (n = 268) of feral cat brain samples collected from around Western Australia, using a hemi-nested PCR directed at the B1 gene. This prevalence was much lower than the seroprevalence detected by MAT in the same study, which was 44 % (n = 25). However, the sample size for serology was much smaller, and from a more restricted geographic distribution than the samples for PCR. The PCR prevalence in this restricted geographic range (Walpole and Perth Metropolitan) was 15 % (n = 38). Additionally, while *T. gondii* can be isolated from the brain in infected cats, it is much more frequently isolated from muscular tissues, specifically tongue and heart (Dubey, 1997). Dubey (1997) isolated *T. gondii* from tongue tissue in 100 % (n = 9) of experimentally infected cats, from heart tissue in 55.6 %, and from brain tissue in 44.4 %, using mouse bioassay. Since the Adams (2003) study only sampled cat brains, this may have resulted in a significant number of false negatives, leading to an inaccurately low prevalence estimate.

Table 1.2: Seroprevalence of *Toxoplasma gondii* antibodies in stray and feral cats sampled from different locations around Australia.

(Adapted from Fancourt and Jackson, 2014)

Location	Test Type	Sample size (n)	Cut-off titre for seropositivity	Number positive	Seroprevalence (%)	Reference
<b>Kangaroo Island, South Australia</b>	DAT	47	≥ 1/4	42	89.4	(O'Callaghan et al., 2005)
<b>Tasmania</b>	MAT	266	≥ 1/64	224	84.2	(Fancourt and Jackson, 2014)
<b>Christmas Island</b>	IFA	25	≥ 1/64	24	96	(Adams et al., 2008)
<i>Victoria</i>						
<b>Melbourne</b>	ELISA	103	Absorbance of ≥ 0.5 at dilution of 1/1000	40	38.8	(Sumner and Ackland, 1999)
<b>Central VIC</b>	IHA	16	1/64	7	43.8	(Coman et al., 1981)
<b>North-western VIC</b>	IHA	59	1/64	8	13.6	(Coman et al., 1981)
<i>Western Australia (WA)</i>						
<b>North-western WA</b>	LAT	42	≥ 1/64	0	0	(Adams, 2003)
<b>Perth</b>	MAT	8	≥ 1/64	6	75.0	(Adams, 2003)
<b>South-western WA (Walpole)</b>	MAT	17	≥ 1/64	5	29.4	(Adams, 2003)
<b>South-western WA (Darling Ranges and west)</b>	IHA	66	Not specified	24	36.4	(Jakob-Hoff and Dunsmore, 1983)
<b>South-western WA (east of Darling Ranges)</b>	IHA	8	Not specified	0	0	(Jakob-Hoff and Dunsmore, 1983)

Abbreviations: DAT = Direct agglutination test, MAT = Modified agglutination test, IFA = Immunofluorescent antibody test, ELISA = Enzyme-linked immunosorbent assay, IHA = Indirect haemagglutination test, LAT = Latex agglutination test

### 1.2.10 Significance of *Toxoplasma gondii* in the environment

Environmental contamination with oocysts is presumed to be the predominant method of transmission of *T. gondii* to non-carnivorous intermediate hosts. This can be due to direct contamination of feed sources (e.g. pasture), contaminated soil, or contaminated water. Contact with soil is increasingly recognised as a risk factor for *T. gondii* infection in large-scale human epidemiological studies (Dabritz and Conrad, 2010). Experimental evidence also suggests that coprophagic invertebrate species (e.g. annelids) may carry infective oocysts within their gut, and act as a source of infection for animals which consume these species (Bettioli et al., 2000b; Frenkel et al., 1975; Ruiz and Frenkel, 1980). Oocysts are highly resistant in the environment, and can withstand freezing temperatures (Frenkel et al., 1975). However, they are susceptible to desiccation and may be destroyed by temperature extremes (Yilmaz and Hopkins, 1972). Oocysts in faecal deposits kept outside in uncovered petri dishes maintained infectivity in mice for 46 days when exposed to environmental temperatures ranging from -6 to 39 °C (Yilmaz and Hopkins, 1972). This was extended to 76 days when the faecal deposits were kept in the shade in the same environment. Furthermore, duration of infectivity was significantly prolonged when the faecal deposits were covered and kept moist, with survival times of 183 days and 334 days in the sun and shade, respectively.

Oocysts can survive for long periods in both fresh and marine water, and experimental evidence has demonstrated survival times of up to 54 months and 24 months, respectively (Dubey, 1998; Lindsay and Dubey, 2009). Contamination of drinking water with oocysts has been implicated in outbreaks of human toxoplasmosis, such as an outbreak on Vancouver Island, Canada, which was thought to be caused by contamination of a municipal water supply with *T. gondii* oocysts shed by cougars (*Felis concolor*) (Aramini et al., 1999; Bowie et al., 1997). In marine environments, proximity to areas with increased freshwater runoff is a risk factor for *T. gondii* infection in southern sea otters (*Enhydra lutris nereis*) (Miller et al., 2002). Additionally, once oocysts enter the marine system, they may be

concentrated in the tissues of filter-feeding molluscs such as snails, oysters and mussels (Arkush et al., 2003; Krusor et al., 2015; Lindsay et al., 2004).

#### 1.2.11 Detecting *Toxoplasma gondii* in the environment

Historical methods for detection of oocysts in environmental samples have predominantly involved direct visualization of oocysts via floatation and microscopy. These techniques are labour-intensive, have poor sensitivity, and also have significant specificity issues, as oocysts of related coccidian genera, *Neospora* and *Hammondia*, may be incorrectly identified as *Toxoplasma* (Dubey, 2010). Mouse bioassays have also been used to test soil samples for the prevalence of oocysts (Coutinho et al., 1982). This method is likely to be sensitive, however it is labour-intensive and requires the euthanasia of large numbers of laboratory animals. Less invasive techniques are desirable. Others have used indirect methods to estimate soil contamination. For example, using *T. gondii* seroconversion rates in a cat population and predicted oocyst shedding based on experimental studies to estimate the level of soil contamination in a region (Afonso et al., 2010).

Advances in molecular techniques have increased the sensitivity of available tests to detect oocysts in environmental matrices and allowed for increased throughput of greater numbers of samples. Lass et al. (2009) detected *T. gondii* oocysts in 18 % (n = 101) of soil samples from urban sites in Poland, using PCR directed at the B1 gene. Sites where cats were frequently observed were selected for sampling and included sand pits, playgrounds, parks, and areas around rubbish dumps. More recently, qPCR directed at the 529 bp highly repetitive element has been used to detect *T. gondii* oocysts in soil samples. L  lu et al. (2011) developed a sensitive method for concentration of oocysts from soil samples and subsequent qPCR analysis. This method has since been used in studies in France and China, and oocysts were identified in 29 % (n = 243) and 30 % (n = 9000) of soil samples, respectively (Gao et al., 2016; Gotteland et al., 2014). Gotteland et al. (2014) randomly selected sampling locations within their study site in rural France, which was centred on a small village and

contained several farms. Gao et al. (2016) focused on an urban city in China but, similarly, sampling sites were also randomly selected, i.e. these studies did not select their sampling sites based on known cat activity. To the author's knowledge there are no published studies that have assessed the level of soil contamination with *T. gondii* oocysts in Australia.

#### 1.2.12 Use of sentinel or indicator species to detect *Toxoplasma gondii*

The epidemiological term, sentinel surveillance, refers to targeted disease surveillance in a select group, the sentinel unit, which is then used to infer disease risk in the greater population of interest (Thrusfield and Christley, 2018). In veterinary epidemiology, sentinel units may be designated herds or flocks. However, free-ranging wildlife (through either passive or active surveillance) are often used as sentinels for disease in domestic species and humans. For example, wild birds, particularly corvids, are used for West Nile Virus surveillance in North America (Eidson et al., 2001). A similar concept exists in ecology, where selected indicator species are used to monitor the health of ecosystems, provide early warning of detrimental environmental changes, and allow researchers to assess the efficacy of different approaches to ecosystem management (Siddig et al., 2016).

Free-range sentinel chicken flocks can be used to estimate soil contamination with *T. gondii* oocysts. They are considered a good indicator as they feed directly from the soil and are capable of being infected with *T. gondii* but rarely develop clinical disease (Dubey et al., 2015). In New England, USA, sentinel chicken flocks on pig farms were able to detect differing levels of environmental contamination with *T. gondii* (Dubey et al., 2015). Geographic variation in *T. gondii* exposure risk has also been assessed using small mammal serological surveys. These are particularly useful when the sentinel, or indicator, species is small, and has a limited home range. These surveys can help to identify environmental factors which affect oocyst distribution and survival. In France, grassland small mammals were found to have a higher *T. gondii* seroprevalence than those trapped in forest habitats, suggesting increased exposure risk in grassland habitats (Afonso et al., 2007). However,

when comparing different host species, it is important to consider differences in host biology (e.g. diet and foraging patterns) which may also affect likelihood of exposure (Afonso et al., 2007).

In the marine environment, southern sea otters have been considered a sentinel species for the emergence of *T. gondii* (typically a terrestrial parasite) (Conrad et al., 2005). Sea otters are considered to be important sentinels, as they feed in the nearshore environment, and share some of the same dietary items as humans (Conrad et al., 2005). Studies on *T. gondii* infection in sea otters have revealed a higher prevalence in animals from locations associated with high freshwater outflow, suggesting that *T. gondii* enters the marine environment via surface water run-off (Miller et al., 2002).

Indicator species have been used in epidemiological investigations into human toxoplasmosis, for example, detection of *T. gondii* seropositive deer mice in riparian environments on Vancouver Island, Canada, indicated that oocysts were deposited close to waterways (Aramini et al., 1999). This provided further evidence that a toxoplasmosis outbreak was associated with a contaminated municipal water supply (Aramini et al., 1999; Bowie et al., 1997). Free-ranging hunted game can also be used as sentinel species to estimate environmental contamination. Furthermore, these data can be used to investigate the public health risks associated with consuming wild game meat. For example, Remes et al. (2018) found that 23.97% of moose hunted for human consumption in Estonia were *T. gondii* seropositive. Additionally, seropositive animals were present in each county sampled, suggesting that oocyst environmental contamination is widespread in Estonia, and that consumption of moose may pose a public health risk (Remes et al., 2018).

### 1.3 Aims of this study

The level of environmental contamination with *T. gondii* at Phillip Island is unknown, and therefore the magnitude of the risk of infection for eastern barred bandicoots released onto the island is

difficult to estimate. This project seeks to define a more precise measure of the risk of exposure to *T. gondii* in released bandicoots, and to develop a better understanding of the epidemiology of toxoplasmosis in free-ranging populations of this species.

The aims of this study are:

1. Establish seroprevalence (via MAT) and prevalence (via qPCR) of infection of *T. gondii* in the definitive host, feral cats, on Phillip Island. Define demographic and spatial variations for these measures.
2. Establish prevalence of infection of *T. gondii* in a eutherian intermediate host, the European rabbit (*O. cuniculus*), on Phillip Island, to determine if this species can be used as a sentinel species for environmental contamination and define spatial variation for this measure.
3. Determine environmental contamination by establishing prevalence of *T. gondii* oocysts in soil at Phillip Island. Define spatial and temporal variation for this measure and determine microhabitat characteristics that are associated with varying levels of contamination.
4. Longitudinal study of survivability, *T. gondii* serological status, and causes of mortality in bandicoots released onto Phillip Island in spring, 2017.

#### 1.4 Argument for relevance and importance of the study

The eastern barred bandicoot is considered extinct in the wild, and it is unlikely that this species will become stable and self-sustaining if it remains only within the current intensively managed reintroduction sites. In order to increase the population sufficiently for it to be able to withstand extinction pressures such as climatic variation, fluctuation in food availability, and disease, larger areas of 'safe' habitat must be identified. This study seeks to gain insights into the epidemiology of *T. gondii* in eastern barred bandicoots at a potential release site and will help to determine the risk

that the parasite presents to the establishment of bandicoot populations. Additionally, this study will determine whether sampling of soil for oocysts, and/or the use of rabbits as a sentinel species, can be used as an indirect measure of risk of *T. gondii* infection in eastern barred bandicoots on Phillip Island. This will provide a reference to which future release sites can be compared. Furthermore, if soil sampling is determined to be effective, this will provide an economical and animal-free means of determining toxoplasmosis risk at new sites. This information will be crucial in assessing the suitability of future reintroduction sites for eastern barred bandicoots. Understanding the epidemiology of this fatal disease is extremely important in ongoing recovery efforts for this extinct in the wild species. Furthermore, while toxoplasmosis is well documented as a cause of fatal disease in Australian marsupials, the epidemiology of the disease in free-ranging populations is poorly understood. Gaining insights into the epidemiology of this disease in eastern barred bandicoots will be informative for conservation efforts involving many other endangered Australian marsupial species which are also susceptible to this disease.

## 2 *Toxoplasma gondii* in feral cats on Phillip Island

### 2.1 Introduction

Cats and other members of the family Felidae are the only known definitive hosts for the protozoan parasite, *Toxoplasma gondii* (Dubey, 2010). Accordingly, understanding the epidemiology of *T. gondii* infection in cat populations is critically important to understanding the transmission of this parasite in environmental systems. As cats maintain life-long antibodies to *T. gondii* after exposure, serology is currently considered the most useful diagnostic technique for epidemiological studies (Dubey et al., 1995a).

Many different serological tests have been developed to detect *T. gondii* infection, including species-specific enzyme-linked immunosorbent assays (ELISA), indirect fluorescent antibody tests (IFAT), and the modified agglutination test (MAT). The MAT is considered a highly sensitive and specific test for the detection of anti-*T. gondii* IgG (Desmonts and Remington, 1980). Reported diagnostic sensitivity and specificity ranges from 82.9 – 97.8 % and 90.29 – 100 %, respectively (Dubey et al., 1995b; Macrì et al., 2009). However, there is no gold standard test for the detection of *T. gondii* in any species. Therefore, calculations of sensitivity and specificity in the literature have largely relied on comparison to other imperfect tests (Dubey et al., 1995b; Gamble et al., 2005; Garcia et al., 2006b; Macrì et al., 2009). Comparison to either cat or mouse bioassay, as a proxy for a gold standard test, is the most common. The bioassay is highly specific; however, it can lack sensitivity (Dubey et al., 1995b). Additionally, most studies have estimated these parameters for the MAT in production animals, such as pigs and sheep (Dubey et al., 1995b; Gamble et al., 2005; Garcia et al., 2006b; Mainar-Jaime and Barberán, 2007). While the MAT is not species-specific, variation in the sensitivity and specificity of this test between host species is likely, and has been suggested by previous studies (Mainar-Jaime and Barberán, 2007). The MAT is frequently considered to be the most sensitive and specific test for detection of *T. gondii* infection in the cat, although the evidence for this appears to

be largely extrapolated from data collected from other hosts (Afonso et al., 2006). Macrì et al. (2009) estimated the sensitivity and specificity of the MAT in cats as 97.8 % and 100 %, respectively. However, these parameters were calculated using IFAT results as the gold standard. The sensitivity and specificity of IFAT in cats has been estimated as 81.0 % and 93.8 %, using a Bayesian approach (Dabritz et al., 2007a). This indicates that the IFAT is an imperfect test, and therefore not appropriate to use as a gold standard.

PCR is a useful test for the detection of *T. gondii* infection for several reasons. Firstly, it is widely considered to have specificities approaching 100 % (Belaz et al., 2015). Furthermore, sensitivity of PCR is also high, but may be variable depending on whether conventional, nested, or real-time PCR is used (Belaz et al., 2015). Selection of tissue(s) and sampling techniques will also influence test sensitivity, given the variation in tissue predilection of *T. gondii* between hosts, and the heterogenous distribution of bradyzoite cysts within tissues (Belaz et al., 2015; Hill et al., 2006). A number of different gene targets and primer sequences have been described, and specificity and sensitivity likely vary between these protocols (Belaz et al., 2015). PCR may be used in situations when sera are not available, or antibody preservation is compromised, for example in wildlife studies when scavenged carcasses are the only available sample set. Furthermore, PCR-based techniques may be expanded to allow sequencing and strain typing, which may be important in detailed epidemiological studies.

In situations where diagnostic tests cannot be compared to a gold standard, Bayesian latent class analysis may be used to determine test parameters such as sensitivity and specificity (Branscum et al., 2005). Furthermore, this approach is the current World Organisation for Animal Health (OIE) recommendation for validation of these parameters in the absence of a gold standard reference test (World Organisation for Animal Health (OIE), 2014). Bayesian analysis has previously been used to

evaluate *T. gondii* diagnostic testing in a variety of species (Dabritz et al., 2007a; Georgiadis et al., 2003; Hillman et al., 2017; Mainar-Jaime and Barberán, 2007).

The aim of this study was to compare two different testing methods for detection of *T. gondii* infection in the cat: a real-time PCR (qPCR) protocol using DNA extracted from proteinase K digested tissues, and the MAT. Although these two methods were testing different manifestations of disease (i.e. detection of *T. gondii* DNA by qPCR and detection of antibodies by MAT), since cats are thought to maintain *T. gondii* antibodies and bradyzoite cysts for life once infected, the comparison was considered appropriate (Dubey, 2010; Dubey et al., 1995a). A Bayesian approach was used to estimate the sensitivity and specificity of each test in cats, and to estimate the true prevalence of infection in the population tested. We hypothesized that the qPCR technique would be highly specific, while the MAT would have increased sensitivity relative to qPCR, at the expense of specificity. This study was conducted on samples from a population of feral cats inhabiting Phillip Island, located in Westernport Bay, Victoria, Australia. We also aimed to determine risk factors associated with *T. gondii* infection in the feral cat population on the island.

## 2.2 Materials and Methods

### 2.2.1 Tissue and blood collection

Blood and tissues were collected opportunistically from feral cats euthanased under animal control programs carried out by authorised officers (authorised by Bass Coast Shire Council) on Phillip Island between June 2016 and November 2017. As all research samples were collected opportunistically from cadavers, ethics approval was not required. Blood was collected by animal control rangers via cardiac puncture post-mortem, placed into serum gel separator tubes and centrifuged for 10 minutes. Serum was collected and stored at -20 °C prior to processing. Carcasses were stored at -20 °C prior to processing. Demographic data, including location, weight, and sex was recorded for each animal. The age of cats was not reliably recorded using an objective measure (e.g. dentition) and

therefore could not be used in downstream statistical analyses. Sample size calculations, shown in Table 2.1, were conducted to determine the number of samples required to estimate the true prevalence, and diagnostic sensitivity and specificity of each test, with 95 % confidence and absolute precision of +/- 5 % (Stevenson et al., 2018).

Table 2.1: Sample size calculations to estimate the sensitivity and sensitivity of the modified agglutination test (MAT) and real-time PCR (qPCR), and true prevalence of *Toxoplasma gondii* infection in the feral cat population (with +/- 5 % absolute precision and 95 % confidence).

Parameter	Estimated value	Sample size required (n)
Specificity MAT	85%	196
Sensitivity MAT	95%	73
Specificity qPCR	99%	15
Sensitivity qPCR	90%	138
True prevalence	90%	138

### 2.2.2 Modified agglutination test

The modified agglutination test was performed using a commercially available kit (*Toxo-Screen DA*, bioMerieux, Mercy l'Etoile, France) with minor adaptations, as per the method developed by Mt Pleasant Laboratory, Department of Primary Industries, Parks, Water, and Environment, Tasmania. Briefly, 25 µl of sample serum was diluted in 175 µl of PBS, and vortexed well to mix, before performing two 1/4 serial dilutions in PBS. Positive and negative control sera were supplied with the kit and used at a dilution of 1/8. 25 µl of each sample dilution, and controls, was transferred to a round-bottom 96-well microtitre plate, and 25 µl of 2-mercaptoethanol was added to each well, resulting in final test serum dilutions of 1/16, 1/64, and 1/256, and 1/16 for the positive and negative control. 25 µl of kit-supplied antigen (formalin treated *T. gondii* tachyzoites) at 1/4 dilution

was added to each well, and the plate shaken gently to mix. The plate was covered with adhesive, or placed within a zip-lock bag, to prevent evaporation and incubated at room temperature for 6 hours. The reaction was considered positive when a layer of agglutinated tachyzoites covering more than half the base of the well was observed. The reaction was considered negative when sedimentation of the tachyzoites formed a small button in the base of the well. Individuals with a titre of  $\geq 1/64$  were considered *T. gondii* seropositive. This cut-off value is consistent with other studies on feral cats in Australia (Adams, 2003; Fancourt and Jackson, 2014). The same observer performed all tests. Results were converted to a binary variable (positive or negative) for analysis.

### 2.2.3 Tissue digestion and DNA extraction

Each carcass was defrosted at room temperature overnight, and tissues were collected the following day. Tissues were either processed immediately or stored at -20 °C. A total of 5 g of tissue, consisting of 1 g each of brain, skeletal muscle (*biceps femoris* m.) and heart, and 2 g of tongue was collected from each carcass. For 31 cats, liver and lung (1 g each) was also collected, and a total of 7 g of tissue was processed. Artificial tissue digestion and DNA extraction was carried out as per Cuttell et al. (2012) with minor adaptations as follows. Tris-EDTA lysis buffer (40mM Tris, 10mM EDTA; Sigma-Aldrich, St. Louis, Missouri, USA) was added to pooled tissue to make up to a total volume of 10 ml and incubated in a water bath at 90 °C for 10 minutes. Samples were cooled to room temperature before the addition of 4mg of Proteinase K (Bioline, London, UK). The samples were incubated overnight at 55 °C, before being homogenized using a tissue homogeniser (T10 Basic Tissue Homogeniser, IKA-Werke, Staufen im Breisgau, Germany) to ensure complete homogenisation. DNA was extracted from the homogenised tissue lysate using either the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), or ISOLATE II Genomic DNA extraction kit (Bioline). After homogenisation, 300 µl of crude lysate was transferred to a 1.5 ml microcentrifuge tube and 300 µl of tissue lysis buffer (Qiagen Buffer AL, or Bioline Buffer G3) was added before incubation at 70 °C for 10 minutes. The sample was centrifuged at 14,000 rpm to pellet cellular debris, and 400 µl of

supernatant was removed to a clean 1.5 ml microcentrifuge tube containing 210 µl of ethanol. At this point, all supernatant was added to the extraction column, and the protocol was followed as per kit instructions. All DNA extractions were assessed for total nucleic acid concentration and purity using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). DNA extraction of the crude tissue lysate was repeated for any samples with significant contaminants likely to affect downstream applications.

#### 2.2.4 Real-time polymerase chain reaction

Genomic DNA extracts were subjected to real-time PCR (qPCR) as described by Lélou et al. (2011) with some minor modifications. Reactions were carried out on a MIC Personal qPCR Cycler (Bio Molecular Systems, Upper Coomera, Queensland, Australia). The 529 bp highly repetitive element (GenBank AF 487550) was targeted by the Taqman probe 5'-6FAM-ACGCTTTCCTCGTGGTGATGGCG-IBFQ-3' and the DNA oligonucleotide primers 5'-AGAGACACCGGAATGCGATCT-3' and 5'-CCCTCTTCTCCACTCTTCAATTCT-3'. As an internal control for PCR inhibition, Equine Herpes Virus (EHV) DNA was added to the reaction mix and targeted by the Taqman probe 5'-HEX-ACGCTTTCCTCGTGGTGATGGCG-IBFQ-3' and the DNA oligonucleotide primers 5'-GATGACACTAGCGACTTCGA-3' and 5'-CAGGGCAGAAACCATAGACA-3'. DNA oligonucleotide primers and probes were synthesized by Integrated Gene Technologies, Singapore. The amplification mixture consisted of 10 µl of GoTaq qPCR Master Mix (Promega, Madison, Wisconsin, United States), 150 nM of each *T. gondii* primer, 150 nM of the *T. gondii* Taqman probe, 40 nM of each EHV primer, 100 nM of the EHV Taqman probe, 1 µl of EHV template DNA, and 2 µl of sample genomic DNA, in a final reaction volume of 20 µl. The reaction mixture was subjected to an initial incubation of 95 °C for 2 minutes, and then 45 cycles of: denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 60 seconds. Negative and positive controls were included in each qPCR run. Results were reported as the Cq value (the number of cycles taken to reach the fluorescence detection threshold). Negative controls consistently exhibited no detectable fluorescence. Each DNA sample was

processed in duplicate, and a sample was considered positive only if there was amplification of each duplicate, and the average Cq was less than or equal to 37 cycles. This Cq cut-off value was determined using a standard curve of known *T. gondii* target DNA concentrations. Target DNA was repeatably detected at concentrations of  $1 \times 10^{-5}$  pg/ $\mu$ l at a Cq = 37; lower concentrations were not reliably detected (data not shown). Results were converted to a binary variable (positive or negative) for analysis.

#### 2.2.5 DNA extraction control

Each cat DNA sample with a negative *T. gondii* qPCR result was amplified using universal eukaryotic primers directed at a highly conserved region of the 18S rRNA gene as a DNA extraction control, to confirm the presence of amplifiable DNA in the sample as described by Fajardo et al. (2008). Primer sequences: forward primer 5'-TCTGCCCTATCAACTTTCGATGG-3', and reverse primer 5'-TAATTTGCGCGCCTGCTG-3'. Each 25  $\mu$ l PCR reaction contained 1x Coral Load Buffer (Qiagen, Hilden, Germany), 100  $\mu$ M dNTPs (Qiagen), 0.4  $\mu$ M of each primer, 1 unit (0.2  $\mu$ l) of HotStarTaq® Plus DNA polymerase (Qiagen), and 2  $\mu$ l of sample DNA. Positive (*T. gondii* genomic DNA) and negative controls were included in each reaction. PCR was performed in a thermocycler (Applied Biosystems, Foster City, California, USA), with the following conditions: initial activation of 95 °C for 5 minutes, 35 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds, and 72 °C for 15 seconds, followed by a final extension step of 72 °C for 2 minutes. PCR products were visualised using gel electrophoresis on 1.5 % agarose gel containing Gel Red® (Biotium, Fremont, California, USA) nucleic acid gel stain.

#### 2.2.6 Logistic regression

Statistical analyses were carried out using Excel 2016 (Microsoft, Redmond, Washington, USA) and Stata (StataCorp, College Station, Texas, USA). The geographical information system application QGIS, version 2.18 (QGIS Development Team, 2018) was used to plot trapping locations.

Multivariable logistic regression modelling was used to assess risk factors associated with *T. gondii*

infection and seroprevalence in cats. To include location data in the model as a categorical variable, an aerial view (Google Maps™, 2018, OpenLayers Plugin, QGIS) of Phillip Island was used to classify the study area into three different land use types: “Park” was land managed by Phillip Island Nature Parks, wildlife sanctuaries, and other public reserves and parks; “Agricultural” was cleared land, both actively farmed and not currently in use; “Residential” was any area with more than 3-4 adjacent buildings/dwellings (Figure 2.1). As an area of high human activity, the Phillip Island racetrack was also included in the “Residential” category. Each putative risk factor (sex, location, season, and weight) was tested independently for statistical significance using univariable logistic regression and only variables statistically significant at  $p \leq 0.20$  were included in multivariable modelling. The final multivariable logistic regression model was selected based on manual reverse step-wise selection, eliminating the most weakly associated of variables that were not statistically significant at  $p \leq 0.05$  at each step, until only statistically significant variables remained. As the age classification provided for each cat was based on subjective opinion alone, it was considered an unreliable variable. Therefore, weight was used in this study as a proxy for age. Weight was analysed as a continuous variable, rather than being classified into different weight categories, to gain the most information from the data. To determine the best way to include weight into the multivariable analysis, a Lowess (Locally Weighted Scatterplot Smoothed) curve and a restricted cubic spline were fit to a scatter plot of the log odds of qPCR positivity vs weight in kilograms (Figure 2.2). This indicated that the relationship was non-linear, and that the most appropriate fit could be achieved by incorporating weight into the regression as a quadratic term centred on 4 kg. The quadratic term for weight was included in multivariable modelling together with a categorical term representing season (with Autumn as the reference category, owing to it having the lowest qPCR positivity). The interaction between weight and season was also tested for statistical significance ( $p \leq 0.05$ ).

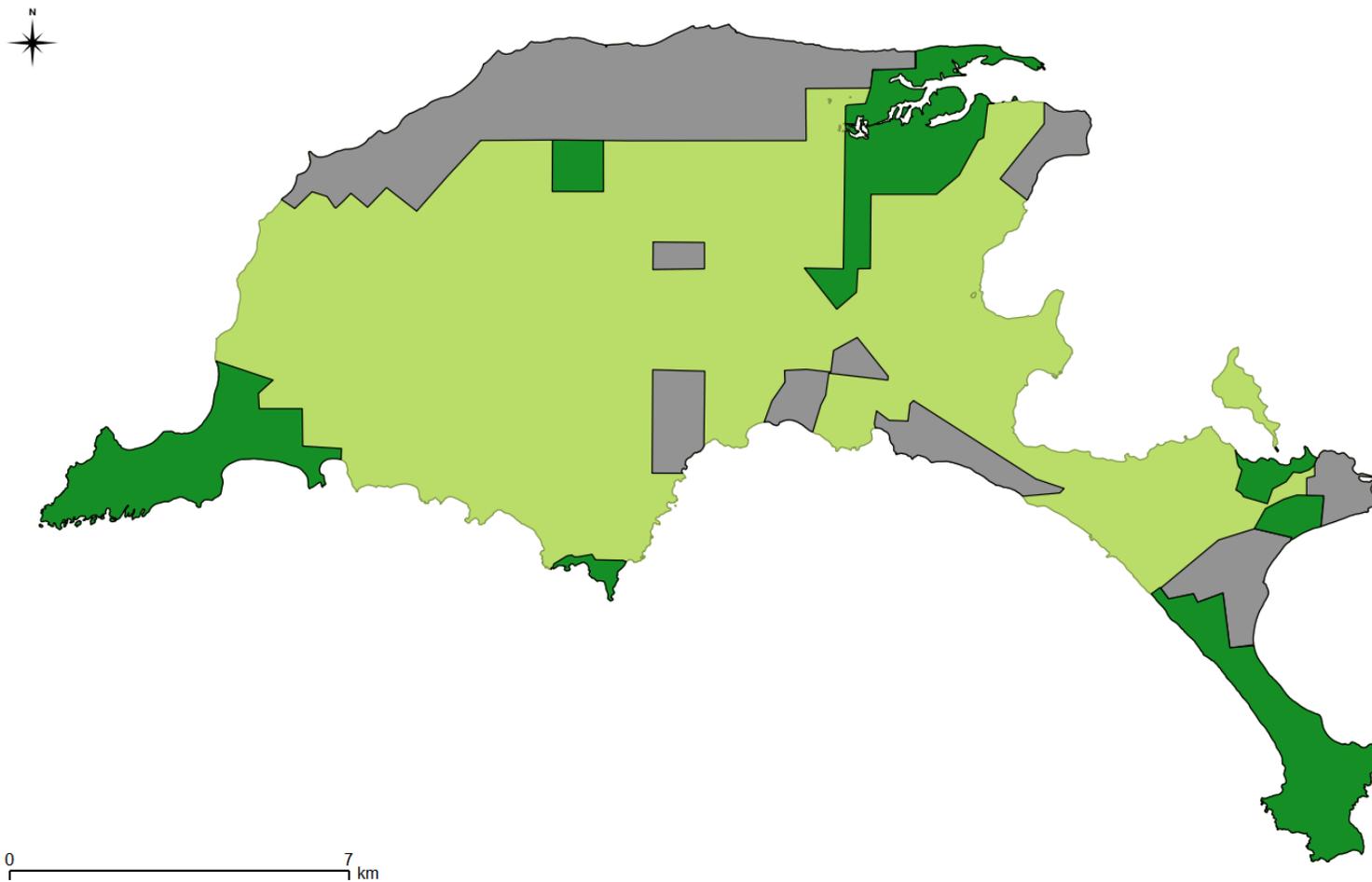


Figure 2.1: Map of Phillip Island showing position and distribution of different location types used in the multivariable regression analysis. Map created using Quantum GIS, version 2.18.

Dark green = "Park", Pale green = "Agricultural", Grey = "Residential"

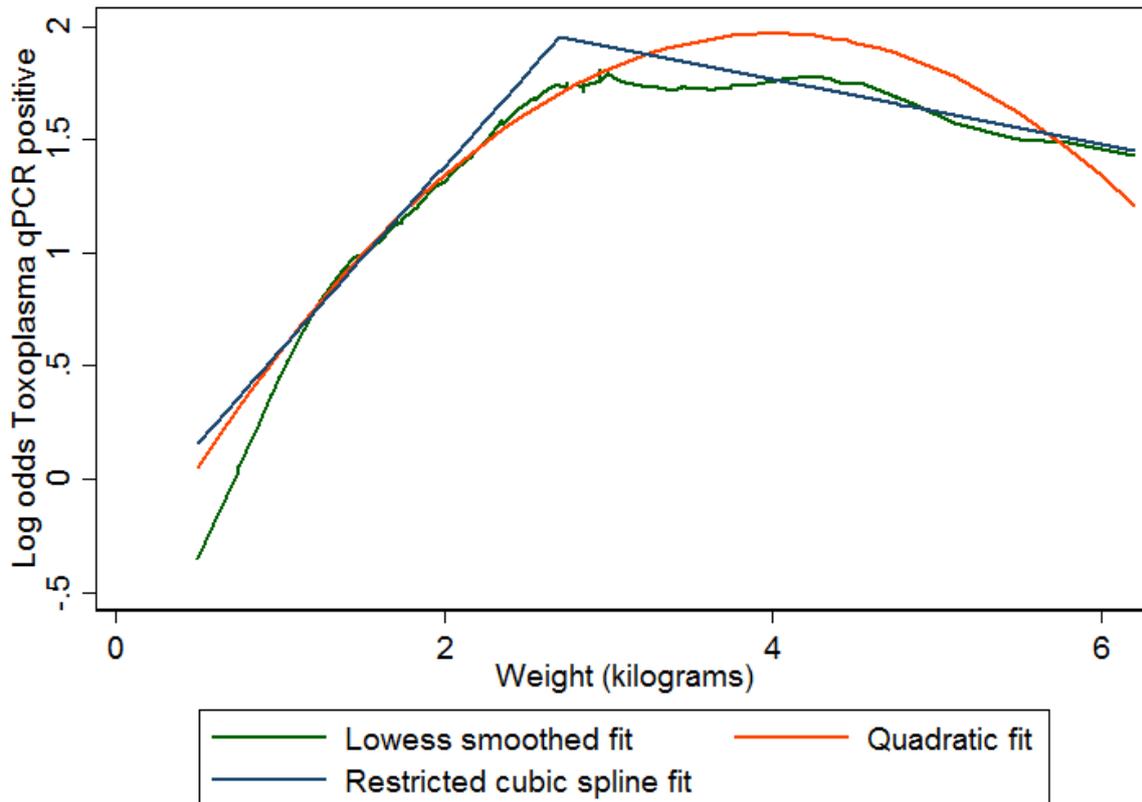


Figure 2.2: Assessing linearity of the relationship between the log odds of *Toxoplasma* qPCR positivity and weight in kilograms.

Legend: These lines of fit clearly show the non-linear relationship between weight (kilograms) and the Log odds of *T. gondii* qPCR positivity. There is a strong positive association, which plateaus at greater weights. The line of fit produced when weight was converted into a quadratic term (red line) fit the data well (green line). Green = Locally Weighted Scatterplot Smoothed (Lowess) fit to a scatter plot of the log odds of *Toxoplasma gondii* qPCR positivity vs weight in kilograms; Blue = restricted cubic spline fit to same data; Red = log odds *T. gondii* qPCR positivity vs weight transformed into a quadratic term.

### 2.2.7 Comparison of the two test methods and Bayesian modelling

The overall agreement of the two tests, Cohen’s kappa coefficient, and prevalence and bias adjusted kappa (PABAK) were calculated using R (R Core Team, 2017), and the epiR statistical package for R (Stevenson et al., 2018). The diagnostic sensitivity and specificity of qPCR and MAT, and the true prevalence of *T. gondii* infection were estimated using a Bayesian approach as described in Branscum et al. (2005), with minor adaptations for two independent diagnostic tests used in the same population. The prior distribution inputs for test Se and Sp were determined by expert opinion.

Three experts in diagnostic parasitology were contacted, and the final values used in the model were the mean of values elicited from the experts (Table 2.2) (A. Koehler, D. Nguyen, R. Traub, personal communication). The prior distribution for true prevalence was based on published data, focusing on those studies reporting *T. gondii* prevalence in feral cats on Australian islands (Adams et al., 2008; Fancourt and Jackson, 2014; O'Callaghan et al., 2005) and expert opinion. The posterior distribution of diagnostic sensitivity, specificity, and true prevalence were obtained using Markov chain Monte Carlo (MCMC) techniques using the WINBUGS® package version 1.4 (Lunn et al., 2000) and R (R Core Team, 2017). The model was run for 40,000 iterations and the first 4,000 'burn in' samples were discarded and chains thinned by a factor to appropriately reduce autocorrelation to < 0.1. The point estimate and 95 % credible interval (CrI) for diagnostic sensitivity, specificity, and true prevalence were reported as the median and 2.5 % and 97.5 % quantiles of the posterior distributions. The tests were assumed to be conditionally independent, because each test was detecting a different manifestation of disease i.e. MAT is an indirect test and detects the host's immune response to infection with *T. gondii* (antibodies) and qPCR detects the organism directly (DNA). In order to check this theoretical assumption, a sensitivity analysis was run using the model for two dependent tests (Branscum et al., 2005). Sensitivity analyses of each prior distribution was also undertaken. The lower credible limit of each of the priors was decreased by 10 % and then by 20 %, while keeping all other model inputs constant. Any change of 5 % or more in the point estimate produced by the model was considered substantial and is reported in the Results section. Additionally, the prior distributions were plotted against the posterior distributions obtained from the model, to allow a visual assessment of the effect of the informative prior distributions on the resulting posterior distributions from the model.

Table 2.2: Prior distributions for Bayesian analysis determined from expert opinion and available literature.

		<b>Mode</b>	<b>Lower limit</b> <b>95 %</b>	<b>Beta</b> <b>distribution</b>	<b>References informing</b> <b>prior distribution</b>
<b>Modified</b> <b>Agglutination</b> <b>Test (MAT)</b>	Specificity	85.3 %	70 %	(23.127,4.813)	Expert opinion
	Sensitivity	91.0 %	78 %	(28.028,3.673)	Expert opinion
<b>Real-time PCR</b> <b>(qPCR)</b>	Specificity	99.7 %	80 %	(13.737,1.038)	Expert opinion
	Sensitivity	94.3 %	82 %	(28.219,2.645)	Expert opinion
<b>True prevalence</b>		89.8 %	76 %	(26.111, 3.852)	(Adams et al., 2008; Fancourt and Jackson, 2014; O’Callaghan et al., 2005) Expert opinion

## 2.3 Results

### 2.3.1 *Toxoplasma gondii* prevalence in feral cats on Phillip Island

A total of 161 feral cats was collected between June 2016 and November 2017. These comprised 91 males and 69 females. Body weight ranged from 0.5 to 6.2 kilograms (mean =  $2.7 \pm 0.11$  standard error). Serum was available from 97 cats. Sex and weight data were not available for one cat. Capture locations spanned across most of the island and *T. gondii* positive individuals were widespread (Figure 2.3).

*Toxoplasma gondii* seroprevalence, as detected by MAT, was 91.8 % (95 % CI 84.6 – 95.8). Modified agglutination tests were titrated out to a maximum dilution of 1/256 for each sample i.e. final end-point titres were not determined. Antibody titres obtained are summarised in Table 2.3. Logistic regression was used to establish if any demographic factors were associated with increased risk of *T. gondii* infection. There was a positive association between *T. gondii* serological status and weight, odds ratio (OR) = 2.3 (95 % CI 1.1 - 4.9; p = 0.029). There was no statistically significant association between sex and *T. gondii* serological status (p = 0.691). The relationship between *T. gondii* serological status and location type or season could not be analysed due to quasi-complete separation of the data and attempts at multivariable modelling resulted in lesser fit than a univariable model for the serological data that only included weight.

Table 2.3: Summary of antibody titres for Phillip Island feral cats sampled from June 2016 to November 2017, as determined by the modified agglutination test (MAT).

<b>MAT titre</b>	<b>Serological status</b>	<b>Number of cats</b>
0	Negative	7
1/16	Negative	1
1/64	Positive	1
1/256*	Positive	88
		<u>Total: 97</u>

\*Each reaction titrated out to a final dilution of 1/256, final end-point titres not obtained.



Figure 2.3: Location and *Toxoplasma gondii* infection status of feral cats trapped on Phillip Island from June 2016 to November 2017. Map created using Quantum GIS, version 2.18, and Google OpenLayers plugin.

Yellow = *T. gondii* qPCR positive, white = *T. gondii* qPCR negative.

*Toxoplasma gondii* qPCR results were available for all cats (n = 161). *Toxoplasma gondii* apparent prevalence, as detected by qPCR, was 79.5 % (95 % CI 72.6 - 85.0). DNA extraction controls confirmed amplifiable eukaryotic DNA was present for all *T. gondii*-negative samples (data not shown). There was no significant association between *T. gondii* infection and sex ( $p = 0.626$ ), location type was initially included but later eliminated from multivariable modelling ( $p = 0.087$ ). Due to the non-linear relationship between weight and the log odds of qPCR positivity (Figure 2.2), weight in kilograms was converted into a quadratic term, centred on 4 kg, for inclusion into the multivariate analysis. Univariable logistic regression models for season and the quadratic term for weight indicated that the effect of these variables on the log odds of being qPCR positive was statistically significant. These variables were included in a multivariable logistic regression model, and the interaction between weight and season was tested and found to be statistically significant (Table 2.4). The effect of weight was statistically significant, with OR = 0.77 (95 % CI 0.62 - 0.94;  $p = 0.011$ ). Weight was included as a parabolic term centred on 4kg, and the protective effect (OR < 1) denotes that the parabola was inverted i.e. the risk of qPCR positivity attained a peak at a body weight of 4kg. Therefore, there was increased risk of qPCR positivity with increasing weight up until 4kg, and only a slight reduction in risk beyond 4 kg within the range of the data. To aid in interpretation of the interaction term presented in Table 2.4, the outputs from the multivariable logistic regression model were plotted as predicted probability of qPCR positivity in cats versus weight in kilograms, for each season (Figure 2.4).

Table 2.4: Odds ratios for continuous and categorical variables included in multivariable logistic regression model for *Toxoplasma gondii* qPCR positivity in cats on Phillip Island.

	Odds Ratio	95 % Confidence Interval	P> z
<b>Season (compared to autumn as reference)</b>			
<i>Summer</i>	1.25	0.15 – 10.13	0.837
<i>Winter</i>	0.71	0.13 – 3.78	0.686
<i>Spring</i>	1.22	0.13 – 11.64	0.863
<b>Weight (incorporated as quadratic term, centred on 4 kg)</b>			
	0.77	0.62 – 0.94	<b>0.011</b>
<b>Season*Weight interaction term (compared to autumn as reference season)</b>			
<i>Summer</i>	1.10	0.80 – 1.51	0.566
<i>Winter</i>	1.41	1.04 – 1.90	<b>0.027</b>
<i>Spring</i>	4.68	0.11 - 201.70	0.421

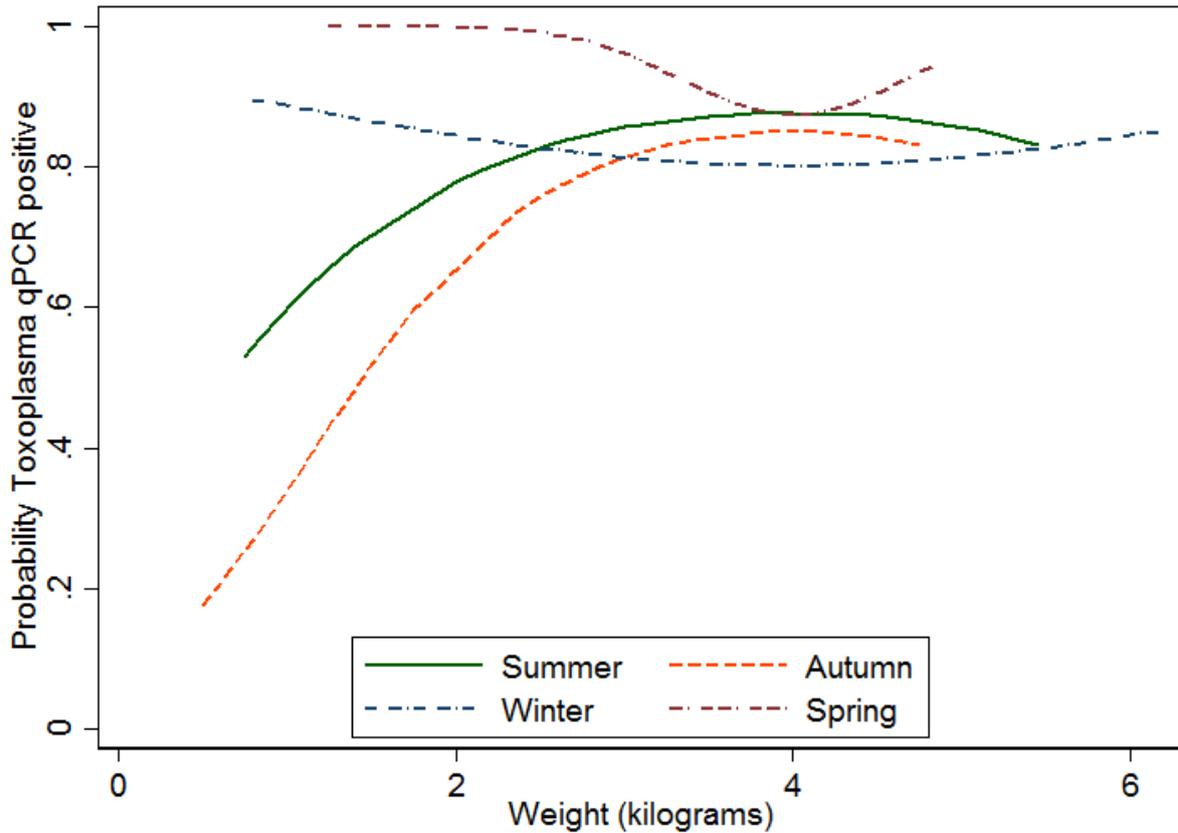


Figure 2.4: Predicted lines of fit for the multivariable logistic regression model plotted as probability of *Toxoplasma gondii* qPCR positivity in feral cats on Phillip Island vs weight (kilograms) for each season.

### 2.3.2 Evaluation of the modified agglutination test and real-time PCR as diagnostic tests for *Toxoplasma gondii* infection in feral cats

The overall agreement between MAT and qPCR was 87.6 %, and Cohen’s kappa coefficient was 0.479 (95 % CI 0.300 – 0.658), suggesting only moderate agreement (Landis and Koch, 1977). However, prevalence and bias adjusted kappa (PABAK) was 0.753 (95 % CI 0.588 - 0.869), indicating substantial agreement between the two tests (Byrt et al., 1993). The estimated diagnostic sensitivity and specificity of the qPCR method used in this study was 90.1 % (95 % Credible Interval [CrI] 83.5 - 95.6) and 96.2 % (95 % CrI 82.2 – 99.8), respectively (Table 2.5). The estimated diagnostic sensitivity and specificity of the MAT was 96.1 % (95 % CrI 91.5 – 98.8) and 82.0 % (95 % CrI 65.1 – 93.3),

respectively. The estimated true prevalence of *T. gondii* infection in the cat population was 90.2 % (83.1 – 95.2). On sensitivity analysis, no substantial changes were observed in the point estimates for the true prevalence of *T. gondii*, the sensitivity and specificity of the qPCR method, or the sensitivity of the MAT. When the lower credible limit of the prior distribution of the specificity of the MAT (SpMAT) was lowered to 50 %, the point estimate of the specificity was reduced to 74.3 % (95 % CrI 44.5 – 95.7). Visual assessment confirmed that posterior distributions for all parameters except SpMAT were informed by the data, rather than overly influenced by the prior distributions (Appendix 1). The plot of prior and posterior distributions of SpMAT suggested that this parameter may have been strongly influenced by the prior distribution inputs. When the data and prior distributions were analysed using the dependent tests model, the 95 % CrI for the correlation terms crossed zero, satisfying the assumption that the tests were conditionally independent and justifying the use of the independent test model.

Table 2.5: Sensitivity and specificity of *Toxoplasma gondii* qPCR protocol and the modified agglutination test, and true prevalence of infection in feral cats on Phillip Island as determined by Bayesian analysis.

		<b>Point estimate (95 % Credible interval)</b>
<b>Modified Agglutination Test (MAT)</b>	<i>Sensitivity</i>	96.1 % (91.5 – 98.8)
	<i>Specificity</i>	82.0 % (65.1 – 93.3)
<b>Real-time PCR (qPCR)</b>	<i>Sensitivity</i>	90.1 % (83.5 -95.6)
	<i>Specificity</i>	96.2 % (82.2 – 99.8)
<b>True prevalence</b>		90.2 % (83.1 – 95.2)

## 2.4 Discussion

The apparent prevalence of *T. gondii* infection in the feral cat population on Phillip Island was 79.5 % and seroprevalence was 91.8 %. Weight was found to be significantly associated both with the probability of cats being *T. gondii* qPCR positive and being seropositive in the MAT. Cats were 2.3 times more likely to be *T. gondii* seropositive with every one-kilogram increase in bodyweight. For the qPCR data, weight was best represented as a parabola with a peak at four kilograms i.e. the risk of being qPCR positive was lower for animals that were less than or greater than four kilograms. Thus, for animals that were less than four kilograms, there was a positive association between weight and the risk of being qPCR positive, i.e. the risk of being infected increased as animals approached a bodyweight of four kilograms. In this study, weight was considered a rough proxy for age. Therefore, these results suggest that up until a cat reaches a certain age (i.e. when they are four kilograms), risk of being infected with *T. gondii* increases with age. This was expected, as once animals are infected with *T. gondii*, bradyzoite tissue cysts are expected to be maintained for the lifespan of that animal (Dubey, 2010). Additionally, cats are considered to maintain lifelong antibodies after a single exposure (Dubey et al., 1995a). Thus, infection prevalence (as measured by both qPCR and MAT) is expected to increase with age (estimated by weight). However, the non-linear relationship between qPCR positivity and weight in this study suggested that the risk of being qPCR positive decreased as weight increased for animals that were greater than four kilograms. This was an unexpected result based on the biological behaviour of *T. gondii*. However, the decrease beyond four kilograms was only slight, and occurred at the edge of the range of the data (number of cats > 4kg = 31, 19 % of total sample set). Furthermore, when the data was fit with a cubic spline with one knot (Figure 2.2), although this appeared to fit the data well visually, the second component of the spline was not statistically significant. Thus, the overall effect is a plateau in the risk of *T. gondii* infection for animals greater than four kilograms. This is clearly represented in Figure 2.4, where there is a flattening off of the slope at the upper end of the weight range in each season, suggesting there is minimal change in probability of *T. gondii* infection at greater body weights.

The final logistic regression model indicated a statistically significant interaction between weight and season (Table 2.4). As seen in Figure 2.4, the probability of *T. gondii* infection increased with increasing weight in summer and autumn. However, in winter and spring, the probability of *T. gondii* infection remained above 80 %, and was relatively constant, regardless of body weight. Although the trend was similar in winter and spring, the interaction term between weight and winter was statistically significant whereas the interaction term between weight and spring was not.

Considering the width of the confidence intervals, this was likely due to the smaller sample size in spring (n = 29), compared to winter (n = 73). These results suggest that in winter and spring, the *T. gondii* challenge is so high for cats that body weight (and by extension, age) ceases to be an important risk factor. Cats born into these seasons are at immediate high risk of infection. Oocyst environmental survival is greatest when the weather is mild and wet (Frenkel et al., 1975; Yilmaz and Hopkins, 1972). High oocyst survival will lead to increased transmission to intermediate hosts and a subsequent increase in *T. gondii* prevalence in cats via predation. Additionally, the time taken for oocysts to sporulate and become infective is dependent on temperature, taking from 2 - 5 days at 15° C to up to 21 days at 11° C (Dubey et al., 1970). Phillip Island has a mild, oceanic, climate with the highest rainfall during winter and spring (Australian Bureau of Meteorology, 2018). This suggests that these are the best months for oocyst survival, as supported by the findings in this study.

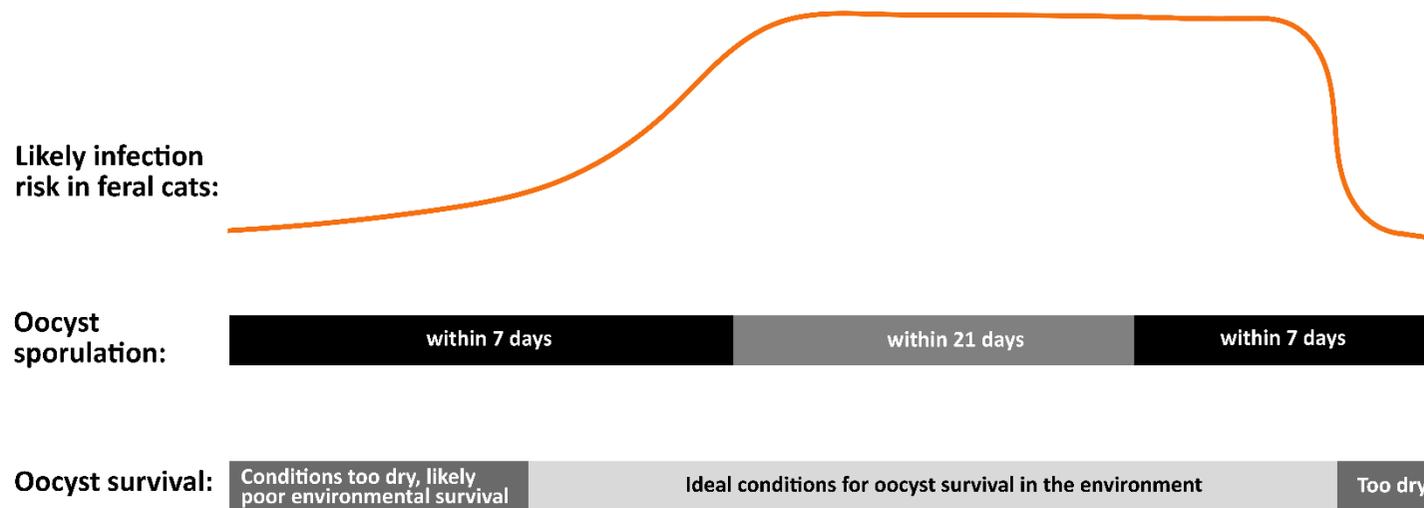
Although rainfall and temperatures in autumn and spring do not differ greatly on Phillip Island, cats trapped in autumn showed the lowest *T. gondii* infection prevalence. This can be explained by a 'lag period' in which the infection prevalence in intermediate hosts is increasing, as a direct result of oocyst contamination, before an increased infection prevalence is observed in the feline population.

The putative seasonal fluctuation in *T. gondii* infection risk in cats on Phillip Island is outlined in Figure 2.5. It is important to note that with the testing methodology used in this study (qPCR and MAT), it was not possible to determine exactly when *T. gondii* positive cats became infected.

Therefore, the association with *T. gondii* infection and season may be more complex than suggested

by the results of this study. In order to further investigate this association, future research approaches could include a longitudinal study of *T. gondii* seroconversion in feral cats on Phillip Island.

Studies elsewhere in the world have also found associations between season and/or climactic factors and *T. gondii* infection in cats. Afonso et al. (2010) found that *T. gondii* incidence risk increased with increasing rainfall in a population of domestic cats. On a broader geographic scale, Afonso et al. (2013) also identified a positive association between the North Atlantic Oscillation (NAO) winter index and *T. gondii* infection. The NAO winter index summarises annual meteorological variations on a continental scale, and higher indices lead to milder, wetter, winters in western Europe (Afonso et al., 2013). Other factors may affect the frequency of *T. gondii* transmission, such as the relative abundance of intermediate hosts, variable oocyst exposure across different prey taxa, and feline dietary preferences. These factors may also vary with season, and therefore act as confounders in determining the relationship between season and *T. gondii* infection prevalence in feral cats. For example, feline dietary preferences have been shown to be affected by both temperature and rainfall and thus will fluctuate with season (Doherty et al., 2015). Future research should be directed at further understanding the relationship between *T. gondii* and season. Determining how infection risk and prevalence in intermediate hosts fluctuates with season may help to develop management guidelines for vulnerable intermediate host species which are susceptible to toxoplasmosis, such as native Australian marsupials.



	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Mean Monthly Maximum (° C)	23.6	23.8	22.6	19.7	16.8	14.4	13.6	14.5	16.1	17.9	19.8	21.8
Mean Monthly Minimum (° C)	13.9	14	12.9	10.7	9.2	7.5	7	7.5	8.3	9	10.5	12
Mean Monthly Rainfall (mm)	35	35.8	54	72.9	80.6	94	90.3	90.7	78.7	76.1	70.6	51.7

Figure 2.5: The effect of seasonal conditions on oocyst sporulation and survival, and *Toxoplasma gondii* infection risk for feral cats on Phillip Island.

Monthly averages obtained from the Australian Bureau of Meteorology (Australian Bureau of Meteorology, 2018).

The Bayesian models used in this study were robust on sensitivity analysis, indicating that the results were primarily driven by the data, rather than being overly influenced by the prior distribution inputs. Only one parameter, the specificity of the MAT, varied substantially on sensitivity analysis. This suggests that this parameter was being overly informed by the prior distribution, rather than the data. Due to time and cost limitations in this study, the final sample size was significantly smaller than the desired sample size to estimate specificity of the MAT (Table 2.1) and this is likely responsible for the reduced precision and variability in this parameter. Regardless, significant variation in the point estimate only occurred when the lower limit of the prior distribution was lowered to 51 %; based on what is known about the specificity of this test in other species, this lower limit was considered very unlikely. Therefore, although the specificity of the MAT should be interpreted with caution, the results of the Bayesian analysis in this study are considered to be valid.

Despite the high overall agreement between the MAT and qPCR results (87.6 % overall agreement), Cohen's kappa (0.479), indicated only moderate agreement between the two tests. However, situations where disease prevalence is very high, as in this study, can drive the kappa statistic down (Byrt et al., 1993). Therefore, in these settings, it is best to use PABAK which adjusts for this effect (Byrt et al., 1993). PABAK in this case was 0.753, and thus indicated there was substantial agreement between the two tests. Each instance of test disagreement between the MAT and qPCR is discussed further as follows. One cat was identified as MAT-negative but qPCR-positive, however, this can be explained if the cat was recently infected. A detectable IgG response to *T. gondii* develops between 14 and 21 days post-infection in the cat (Dubey et al., 1995a). Therefore, if a cat was sampled during acute infection, *T. gondii* tachyzoites would be expected to be detected by qPCR, however, IgG antibodies would not yet be present to result in a positive MAT. Eleven individuals were MAT-

positive but qPCR-negative, these discordant results suggest that the qPCR had a lower sensitivity than the MAT. Concordantly, Bayesian methods estimated the sensitivity of the qPCR method in this study as 90.1 %, compared to 96.1 % for MAT. As the distribution of bradyzoite tissue cysts is likely to be random within predilection sites (i.e. muscle and neural tissue), it is possible to sample tissues from an infected animal without these samples incorporating a tissue cyst. The protocol in this study used a large quantity of tissue (5 g) and included the optimal predilection sites (heart, tongue, brain, and skeletal muscle) to maximise test sensitivity. Alternatively, the eleven MAT-positive, PCR- negative samples may represent MAT false positives. In this study, the specificity of the qPCR was estimated at 96.2 %, compared to 82.0 % for the MAT, confirming our hypothesis that qPCR would have greater specificity than the MAT. Real-time PCR targets a unique sequence of DNA only present in *T. gondii*, leading to the inherently high specificity of this technique. In contrast, the MAT will detect the presence of any antibodies able to bind to *T. gondii* tachyzoites. Therefore, cross-reactivity with closely related apicomplexans can occur and this is documented in the literature. Intermediate hosts infected with *Hammondia hammondi* are known to exhibit serological cross-reactivity in the MAT, and monoclonal antibodies directed against *T. gondii* antigens have been shown to bind to *H. hammondi* *in vitro* (Dubey and Sreekumar, 2003; Riahi et al., 1998). Although this cross-reactivity is not well documented in the literature for cats, there are instances where *T. gondii* has not been isolated from MAT positive cats, but these individuals have been found to be shedding *H. hammondi* (Dubey et al., 2013). Future work should attempt to ascertain the prevalence of *H. hammondi* infection in this population and investigate the importance of MAT cross-reactivity further in cats. Studies have also documented PCR cross-reactivity between *H. hammondi* and *T. gondii* (Schaes et al., 2008a). However, since *H. hammondi* does not form tissue cysts in cats, this is not possible in this study (Dubey and Sreekumar, 2003). Thus, the advantage of qPCR is its superior specificity for

detecting *T. gondii* infection in cats when compared with the MAT. The main disadvantage to this qPCR method is that it cannot be used for antemortem epidemiological screening.

However, this is unlikely to be a significant issue in studies investigating *T. gondii* epidemiology in feral animal populations.

The estimated true prevalence of *T. gondii* infection in the cat population was 90.2 % (95 % CrI 83.1 – 95.2). This high prevalence of infection is consistent with studies conducted on other Australian islands (Adams et al., 2008; Fancourt and Jackson, 2014; O’Callaghan et al., 2005). *Toxoplasma gondii* is likely to be of particular importance in Australian ecosystems, as native Australian marsupials are thought to be exquisitely sensitive to fatal toxoplasmosis (Dubey, 2010). Phillip Island is a significant ecotourism destination in Victoria, and provides habitat for several important species, such as the critically endangered eastern barred bandicoot (*Perameles gunnii*), which has recently been released onto the island, and the Australian fur seal (*Arctocephalus pusillus*), which breeds off-shore. These species, and others inhabiting the island, are likely to be susceptible to toxoplasmosis (Bettioli et al., 2000a; Donahoe et al., 2014). Thus, understanding the epidemiology of *T. gondii* on Phillip Island will be essential to managing this disease, and maintaining the island’s status as a ‘safe-haven’ for wildlife.

Additionally, there is a paucity of literature regarding the genotypes of *T. gondii* infecting feral cats within Australia. Given that genotype may be associated with virulence (Dubey, 2010), an important future direction of this research will be to determine the genotypes of *T. gondii* present in this cat population by DNA sequencing analysis. This will help to provide greater understanding about the epidemiology of different *T. gondii* genotypes in Australia.

## 2.5 Conclusion

This cross-sectional study determined the apparent prevalence and seroprevalence of *T. gondii* infection in feral cats on Phillip Island, Victoria, using the MAT and a Taq-Man probe-based qPCR. Bayesian modelling of this data was then utilized to predict the true prevalence of *T. gondii* infection in this population. The true prevalence of *T. gondii* infection in feral cats on Phillip Island was found to be very high, indicating that toxoplasmosis may pose a significant risk to native wildlife inhabiting the island. Furthermore, *T. gondii* infection risk in cats was found to be associated with season. This finding may be important in developing a deeper understanding of the epidemiology of this parasite in Australian ecosystems, and the risk that it poses to native species that are susceptible to toxoplasmosis.

## 2.6 Appendices

### Appendix 1: Prior distributions and posterior distributions obtained from Bayesian modelling

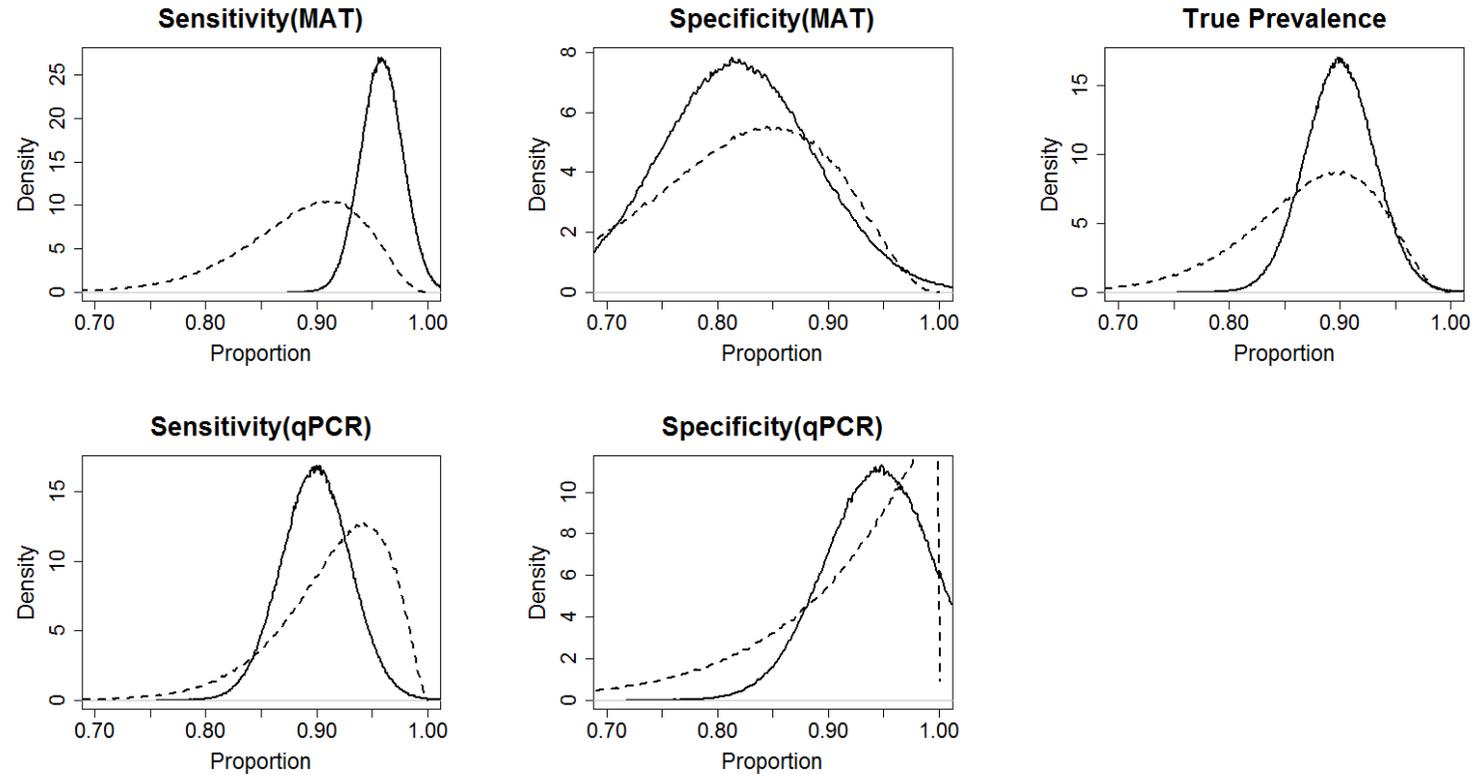


Figure 2.6: Plots of prior distributions and posterior distributions obtained from Bayesian modelling for each parameter. Dashed lines = prior distributions, solid lines = posterior distributions

## 3 Estimating the environmental load of *Toxoplasma gondii* on Phillip Island

### 3.1 Introduction

Many globally significant parasites have an environmental phase to their lifecycle.

Understanding environmental transmission, and developing accurate measures of parasite contamination, is imperative to epidemiological studies attempting to quantify the risk posed by these parasites. *Toxoplasma gondii* is one such example and has important implications for both human and animal health. Infection with *T. gondii* does not typically cause disease in mature, immunocompetent individuals of most species (Dubey, 2010). However, it is known to cause abortion and severe multi-systemic disease in neonates, immunocompromised animals, and in many native Australian marsupial species.

Infection with *T. gondii* can occur via several pathways (discussed in detail in Chapter 1).

Environmental transmission is likely to be the most important pathway in domestic animals and is increasingly recognised as a significant source of exposure in humans (Dabritz and Conrad, 2010). Oocysts are the environmental stage of the parasite and are shed in the faeces of the definitive hosts, members of the family Felidae - primarily *F. catus*, the domestic cat (Dubey, 2010). An infected feline host typically sheds oocysts for around eight days, and the number of oocysts produced in a single infection has been estimated at 50 million (Dabritz et al., 2007b). Once sporulated, oocysts are highly resistant and can survive for up to 18 months in soil (Frenkel et al., 1975). Survival is dependent on environmental conditions, such as humidity and temperature, and is greatest when the climate is mild and wet (Frenkel et al., 1975; Yilmaz and Hopkins, 1972). Little is known about how oocysts may be dispersed through soil from a cat defaecation site. In freely-draining soil, oocysts may be disseminated further by

rainwater surface run-off (Aramini et al., 1999). In addition, feline behaviour and defaecation site preference likely contributes to the heterogenous distribution of infectious oocysts in soil (Afonso et al., 2008).

Historically, identification of oocysts within environmental matrices has proved challenging (Dumètre and Dardé, 2004). In terrestrial environments, this is largely due to the low concentration of oocysts in soil, and limitations with the sensitivity of available techniques. When environmental contamination cannot be measured directly, intermediate hosts may be used as indicator species and *T. gondii* exposure (e.g. seroprevalence) can be used to estimate environmental load. Indicator species can be used to identify geographic variation in contamination, particularly when the indicator species is small, and has a limited home range. This can help to identify environmental factors which affect oocyst distribution and survival.

More recently, direct PCR-based molecular methods have been developed to detect oocysts in soil, increasing the sensitivity of detection and allowing a higher throughput of samples (Lélu et al., 2011). These studies have confirmed the heterogenous distribution of oocysts in soil; in one urban study *T. gondii* was only detected in soil from cat defaecation sites (Afonso et al., 2008). In a rural study, on the other hand, oocysts were more likely to be detected at known defaecation sites, but also frequently found at random sites (Simon et al., 2017). In addition, oocysts were detected with decreasing frequency as distance from farm buildings and villages increased (Gotteland et al., 2014)

Eastern barred bandicoots, *Perameles gunnii*, are known to be susceptible to fatal toxoplasmosis (Bettiol et al., 2000a). This bandicoot species feeds primarily on terrestrial invertebrates, such as annelids, which are accessed by small, shallow (3-5cm deep) diggings in

the soil (Mallick et al., 1997). As a result, eastern barred bandicoots may become infected with *T. gondii* via the ingestion of oocysts, either through ingestion of contaminated soil or plant material during the foraging process, or invertebrates acting as transport hosts. Therefore, estimating soil contamination with oocysts may be able to inform *T. gondii* exposure risk in this species. Phillip Island, Victoria, Australia has been proposed as a potential release site for eastern barred bandicoots, due to the absence of introduced European red foxes (*V. vulpes*) which can heavily predate this species, and the presence of appropriate habitat and food sources (Parrott et al., 2017). Phillip Island has a significant feral cat (*F. catus*) population, and approximately 90 % of these are infected with *T. gondii* (see Chapter 2). Accordingly, toxoplasmosis is likely to pose a significant risk to the successful establishment of *P. gunnii* populations on Phillip Island.

This study combined two different techniques to attempt to estimate environmental contamination with *T. gondii* on Phillip Island, to evaluate risk for future release of eastern barred bandicoots. European rabbits, *Oryctolagus cuniculus*, were examined as an indicator species, and prevalence of *T. gondii* infection was determined by real-time PCR (qPCR) on tissue samples. Direct detection of oocysts in soil was also attempted using a qPCR-based assay. Furthermore, this study aimed to identify the distribution of *T. gondii* contamination on the island, and to help elucidate reasons for any observed geographic variation, and/or association with microclimate and habitat type.

## 3.2 Materials and Methods

### 3.2.1 Soil sampling study sites and sampling design

The soil study was undertaken during September and October 2017, at two sites on Phillip Island, Victoria, Australia. The climate is oceanic, with an annual mean rainfall of 713 mm; the

average monthly minimum and maximum temperatures range from 6.9 - 14.1 °C (annual mean minimum 10.2 °C), and 13.7 - 23.8 °C (annual mean maximum 18.8 °C), respectively (Australian Bureau of Meteorology, 2018). Spring was chosen for the sampling period due to the high rainfall and mild temperatures, as this was expected to result in the highest oocyst environmental survival. The first site, the Summerland Peninsula (38°30'37.0"S, 145°08'33.2"E), is approximately 424 ha in size. It is located at the western extremity of the island and was the planned site for release of eastern barred bandicoots. The peninsula is a coastal plateau of predominantly *Poa* spp. grassland and *Melaleuca* spp. woodland, surrounded by beaches, dunes, and rocky cliffs. The area also includes a wetland centred around a freshwater body, Swan Lake. At the time of the study, the feral cat population on Summerland Peninsula was estimated at 1.1 cats/km<sup>2</sup> using a mark-recapture method and camera trap data (A. Rendall, unpublished data). A second site, Cape Woolamai (38°33'10.9"S, 145°21'06.8"E), an approximately 467 ha headland on the south-eastern coast of the island, was chosen as a comparison site. Cape Woolamai has an elevation of 112 m above sea level and is one of the highest points on the island. The environment includes *Banksia* spp. woodland, coastal tea-tree (*Leptospermum* spp.), dunes, beaches and cliffs. This site had an estimated cat density of 4.2 cats/km<sup>2</sup> at the time of the study, calculated using the same technique as above (A. Rendall, unpublished data).

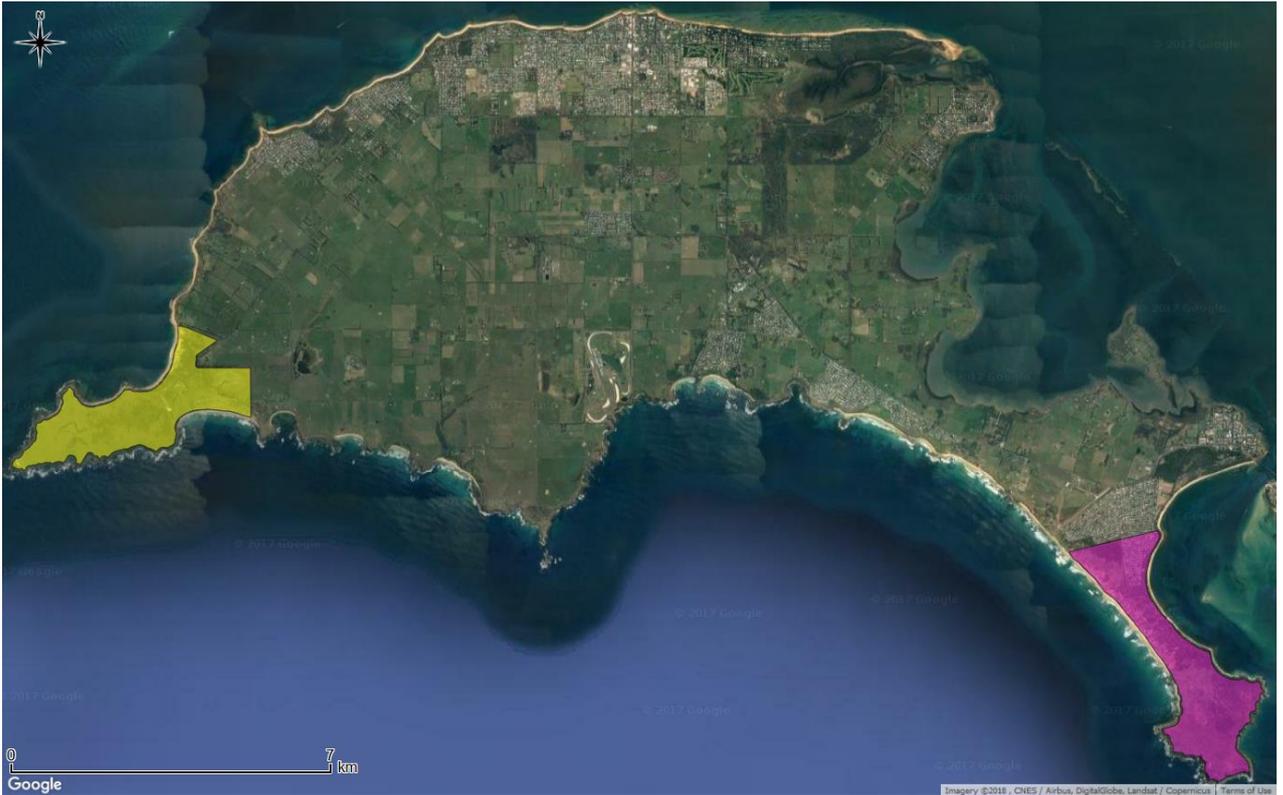


Figure 3.1: Location of soil sampling sites on Phillip Island.

Yellow = Summerland Peninsula; magenta = Cape Woolamai. Satellite image obtained from Google Maps via QGIS OpenLayers plugin. Map created using Quantum GIS, version 2.18.

The geographic information system application QGIS version 2.18 (QGIS Development Team, 2018) was used to design polygons of each study site (Figure 3.1). The boundaries of the study sites were determined by the borders demarcating reserve areas belonging to Phillip Island Nature Parks. No private land was included in this aspect of the study. For safety and accessibility reasons, a 50 m buffer was excluded from the edge of each study area polygon, to prevent any sampling sites from being located on cliffs, rocks, or beach. A satellite image of the area (Source: Google™ Maps, via QGIS OpenLayers plugin) was used to determine the presence of any man-made structures or water bodies. These areas were also removed from each study area polygon. A random-origin grid was generated within each study site polygon using QGIS, and samples were collected from each grid vertex. The number of samples taken at each site

(e.g. number of grid vertices) was restricted by both financial and temporal limitations. In addition, due to the lack of studies investigating the presence of *T. gondii* oocysts in soil samples in Australia, the expected probability of oocyst detection was difficult to estimate. Based on data from international studies, the estimated feral cat density, and the prevalence of *T. gondii* infection in the feral cat population, it was predicted that the probability of detection of oocysts in soil would range from 10 – 15 % of samples. The sample sizes required to estimate a proportion of 10 or 15 % (with 95 % confidence and +/- 0.05 precision) were calculated as 139 and 196, respectively (Sergeant, 2018). A 150 m x 150 m random-origin grid generated over the Summerland Peninsula polygon yielded 157 vertices (see Figure 3.2); this was considered an appropriate sample size, based on the calculations above, and practical limitations on the sampling. A 250 m x 250 m grid, yielding 57 vertices, was designed for Cape Woolamai (Figure 3.2). Cape Woolamai was not the primary site of interest, and therefore the less extensive coverage of the area was considered adequate. Grid vertices were labelled numerically, with a two-letter designation of “SP” or “CW” depending on study site e.g. first grid vertex on Summerland Peninsula = “SP1”. Approximately 100 g of soil was collected at each grid vertex as follows: 10 sub-samples of approximately 10 g of soil were collected over an area of 1 m<sup>2</sup>, at a depth of no more than 5 cm. Samples were collected with a shallow trowel (cleaned with 10 % bleach between each sample) and stored in snap-lock bags. A qualitative description of soil composition and environment type was recorded for each location. Soil samples were collected in September 2017 and stored at 4 °C for a maximum of two months prior to processing.

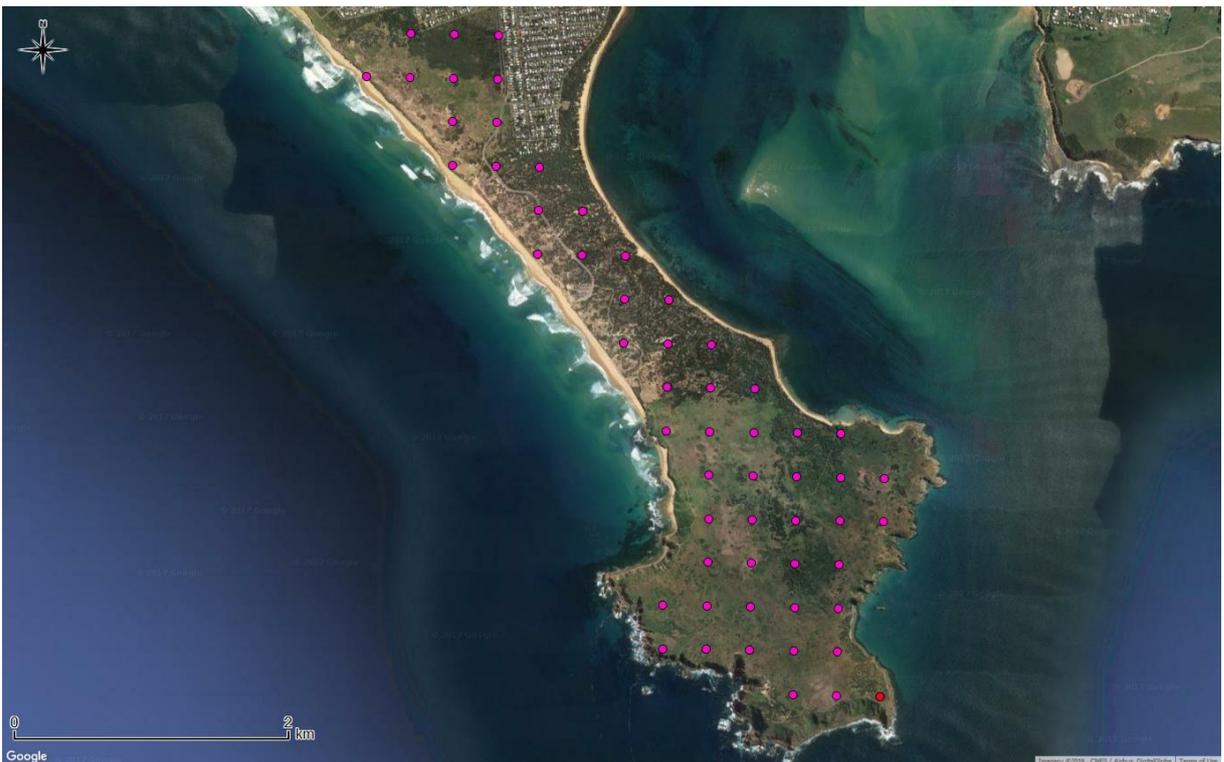


Figure 3.2: Soil sampling grids at Summerland Peninsula and Cape Woolamai.

Above: Summerland Peninsula; Below: Cape Woolamai. Red points signify grid vertices that were inaccessible during soil collection in September and October 2017.

### 3.2.2 Oocyst recovery

*T. gondii* oocysts were isolated from soil using the following technique (adapted from Lélou et al., 2011). Soil from each sampling location was processed as two 5 g duplicates, designated “A” and “B” in addition to each vertex label, e.g. “SP1A”. The remainder was stored at 4 °C for later use, if required. Each 5 g sample was placed in a 50 ml conical centrifuge tube, 10 ml of deionised water was added, and the sample was vortexed briefly on low speed to allow for dispersal. The dispersion step differed from that reported by Lélou et al. (2011) in that the duration and speed of vortexing was decreased. This step was altered to reduce the potential impact of sand on oocyst recovery proposed by Lélou et al. (2011). In a new tube, 20 ml of cold sugar solution (specific gravity 1.2) was then overlaid with the sample solution and centrifuged at 1,500 x g for 20 minutes. The supernatant containing the interface between the water and the sugar solution was collected (approx. 15 ml) and 35 ml of deionised water was added. The tube was centrifuged at 1,500 x g for 20 minutes and 1 ml of sediment was retained. This sediment was transferred to a 1.5 ml microcentrifuge tube and stored at -20 °C until DNA extraction.

### 3.2.3 Validation of oocyst recovery technique

*Toxoplasma gondii* oocysts were not available to experimentally inoculate soil samples and validate the oocyst recovery technique. As a proxy for *T. gondii* oocysts, *Eimeria acervulina* oocysts were used to inoculate soil samples and validate the technique. *Eimeria acervulina* oocysts have similar properties to *T. gondii* oocysts and both species are expected to float in solutions of SG > 1.2 (Carvalho et al., 2011; Dubey et al., 1970; Dumètre and Dardé, 2004). Soil samples from Phillip Island were inoculated with approximately 100,000 oocysts. Inoculated soil samples were either refrigerated at 4 °C in lidded containers for 7 days, exposed to environmental temperatures in open-topped containers for 7 days, or used immediately.

Oocyst recovery was performed according to the technique described above, and oocysts were visualised by microscopy of the final sediment obtained. *Eimeria acervulina* oocysts were isolated from all experimentally inoculated samples. Oocyst recovery was subjectively assessed as moderate to high, however, oocyst recovery percentages were not obtained.

#### 3.2.4 DNA extraction from soil sediment samples

Sediment obtained from the oocyst isolation step was thawed at room temperature and centrifuged at 14,000 rpm for 1 minute. Excess supernatant was removed, and the pellet resuspended in 500 µL of supernatant. Total DNA was extracted from the resuspended pellet using the ISOLATE Faecal DNA extraction kit (Bioline, London, UK) per the manufacturer's instructions, including processing in a high-speed bead beater (Mini-Beadbeater-24, Biospec, Bartlesville, Oklahoma, USA) for 2 minutes to ensure rupture of oocysts. Samples were incubated for 5 minutes at room temperature after the addition of DNA elution buffer to increase DNA yield, as recommended by the manufacturer. DNA was eluted in 50 µL of elution buffer and stored at -20 °C prior to downstream applications.

#### 3.2.5 Rabbit tissue collection, dissection and DNA extraction

Rabbit heads were collected opportunistically from feral rabbits euthanased under feral animal control programs on Phillip Island from August 2016 to November 2017. Where known, the location of collection was recorded. Rabbits were collected from five sites on Phillip Island, Summerland Peninsula, Cape Woolamai, "McFees", "Cleelands", and "Cape Kitchen". McFees, Cleelands, and Cape Kitchen are all private land. McFees is approximately 100 ha of cattle grazing land; Cleelands is a sheep and cattle grazing property of approximately 200 ha size; and Cape Kitchen is unmanaged farm land of approximately 50 ha size, adjacent to the Phillip Island airstrip. Cape Kitchen has anecdotally high cat and rabbit densities, however, no

calculated population density estimates were available for any of the private sites (S. Murphy, personal communication). All heads were stored at -20 °C prior to processing. They were defrosted overnight at room temperature for dissection. The following day, brain, skeletal muscle (*masseter* m.), tongue, and one eye were collected from each head. All equipment was cleaned with 10 % bleach and rinsed thoroughly with water between individuals. Any tissues not used immediately were stored at -20 °C.

A total of 5 g of tissue from each rabbit (brain 2 g, tongue 1 g, masseter muscle 1 g, eye 1 g) was subjected to an overnight tissue digestion with Proteinase K (Bioline, London, UK), as described in detail in Chapter 2, Section 2.2.3). DNA was extracted from the homogenised tissue lysate using the ISOLATE II Genomic DNA extraction kit (Bioline), with minor modifications to the manufacturer's protocol as described in Chapter 2.

### 3.2.6 Real-time Polymerase Chain Reaction

Soil and rabbit DNA samples were subjected to qPCR as described by Lélou et al. (2011) with some minor modifications. This protocol is described in detail in Chapter 2, Section 2.2.4, of this thesis. Negative and positive controls were included in each qPCR run. Results were reported as the C<sub>q</sub> value (the number of cycles taken to reach the fluorescence detection threshold). Negative controls consistently exhibited no detectable fluorescence. Each DNA sample was processed in duplicate, and samples were only considered positive if both duplicates were amplified, and the average C<sub>q</sub> was less than or equal to 37 cycles. Results were converted to a binary variable (positive or negative) for analysis.

### 3.2.7 DNA extraction control

Each DNA extract (from 3.2.4 and 3.2.5) with a negative *T. gondii* qPCR result was amplified using universal eukaryotic primers directed at a highly conserved region of the 18S rRNA gene as a DNA extraction control, to confirm the presence of amplifiable DNA in the sample as described in detail in Chapter 2, Section 2.2.5, of this thesis.

### 3.2.8 Statistical methods

Prevalence estimates and confidence intervals for *T. gondii* infection in rabbits, and oocyst contamination of soil samples, were calculated using EpiTools epidemiological calculators (Sergeant, 2018).

## 3.3 Results

### 3.3.1 Presence of *Toxoplasma gondii* in soil samples

Despite the care taken in the experimental design to avoid obstacles when constructing grids at each study location, some grid sites proved to be inaccessible once in the field (e.g. due to expanded water bodies, restricted areas, and cliffs) and no soil was collected at these sites. Soil samples were thus collected from 150 out of 157 and 56 out of 57 vertices on Summerland Peninsula (SP) and Cape Woolamai (CW), respectively (Figure 3.2). A total of 412 soil DNA samples, from 206 sites, were subsequently analysed. Predominant environment types at sample sites on Summerland Peninsula were *Poa* spp. grassland and scrub, compared to scrub and ground cover or grass on Cape Woolamai. Clay loam and sandy loam soils were the most common soil type sampled at the Summerland site, compared to loam and sand at Cape Woolamai. Except samples SP55B and SP128B, all samples were amplified by 18S rRNA eukaryotic PCR, confirming the presence of amplifiable eukaryotic DNA (data not shown). As

the duplicate samples SP55A and SP128A were both 18S rRNA positive, sites SP55 and SP128 were not excluded from downstream statistical analysis. Equine Herpes Virus DNA internal control was reliably amplified from each site duplicate by qPCR at consistent Cq values, confirming the absence of PCR inhibitors in all DNA samples. *Toxoplasma gondii* DNA was not detected in any of the soil samples from either site. Oocyst prevalence was calculated as 0 % at Summerland Peninsula (95 % CI 0 – 2.5), and 0 % at Cape Woolamai (95 % CI 0 – 6.4).

### 3.3.2 *Toxoplasma gondii* prevalence in feral rabbits

A total of 134 feral rabbits was collected from August 2016 to November 2017, but location was only recorded for 79 rabbits. Fourteen rabbits were positive for *T. gondii* DNA by qPCR, resulting in an overall prevalence of 10.5 % (95 % CI 6.3 - 16.8) (Table 3.1). Eight of the positive rabbits were from Cape Kitchen, at a prevalence of 21.6 % (n=37; 95 % CI 11.4 – 37.2) (Figure 3.3). The exact location for the other six positive rabbits was not known, but they were known not to be from Summerland Peninsula (S. Murphy, personal communication). Rabbits from Summerland Peninsula had a *T. gondii* prevalence of 0 % (n=38; 95 % CI 0 – 9.2). This was significantly different to the prevalence at Cape Kitchen (Fisher's exact test, two-tailed p-value = 0.002) (Figure 3.3). Data from McFees, Cleelands, and Cape Woolamai were not included in statistical comparisons due to the extremely small sample sizes at these locations. DNA extraction controls confirmed amplifiable eukaryotic DNA was present for all *T. gondii*-negative rabbit samples (data not shown).

Table 3.1: Prevalence of *Toxoplasma gondii* in rabbits collected from different locations on Phillip Island, as detected by qPCR

Location	Number collected	Number positive	Prevalence (95 % CI)
Cleelands	1	0	N/A*
McFees	1	0	N/A*
Cape Woolamai	2	0	N/A*
Cape Kitchen	37	8	21.6 % (11.4 – 37.2)
Summerland Peninsula	38	0	0 % (0 – 9.2)
Unknown	55	6	10.9 % (5.1 – 21.8)
<b>TOTAL</b>	<b>134</b>	<b>14</b>	<b>10.5 % (6.3 - 16.8)</b>

\* Not calculated, not meaningful due to extremely small sample size.

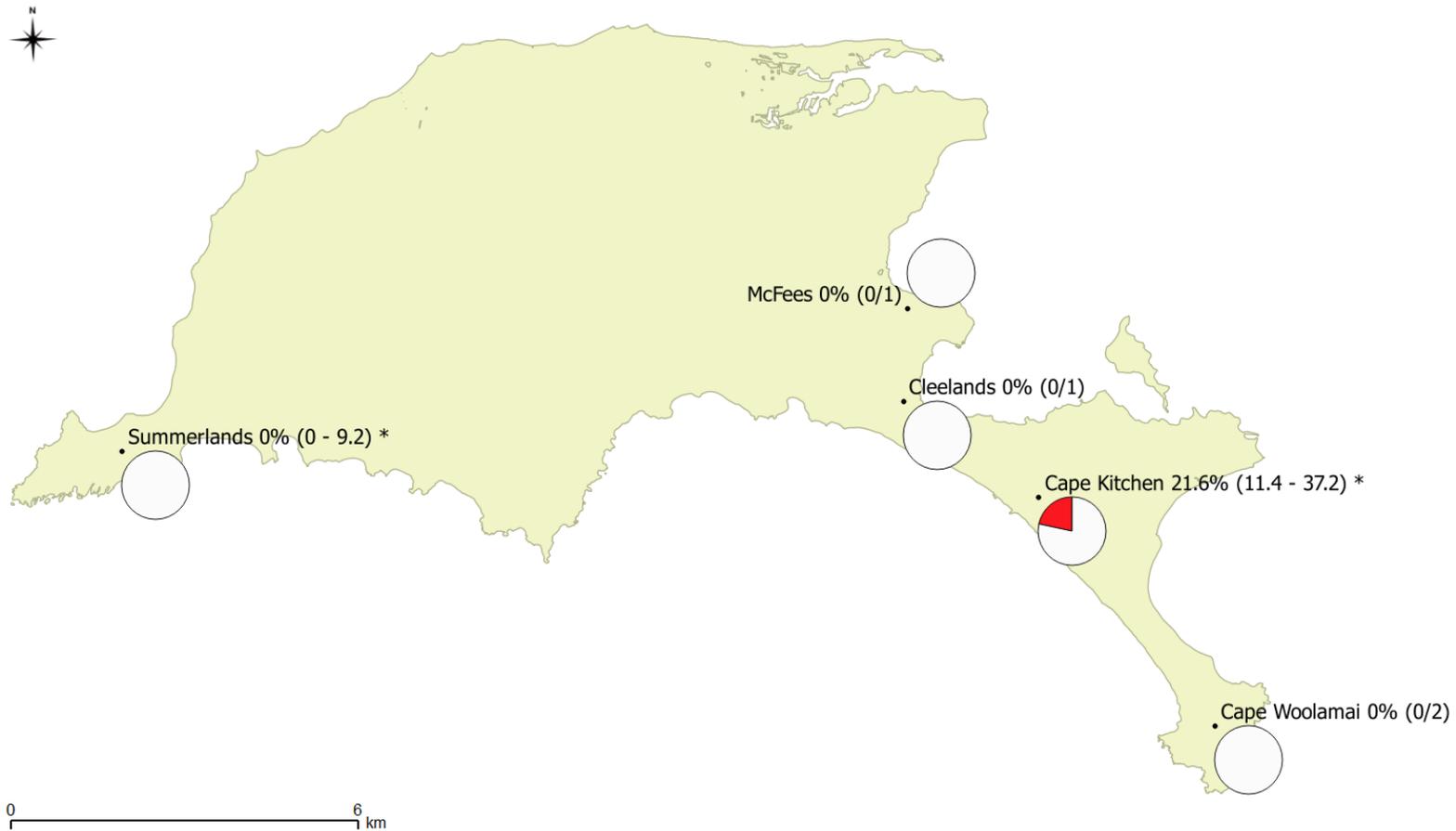


Figure 3.3: Location of feral rabbit collection sites on Phillip Island from August 2016 to November 2017, and proportion of *Toxoplasma gondii* qPCR positive rabbits from each site.

Brackets indicate 95 % confidence interval. Not calculated for McFees, Cleelands, or Cape Woolamai due to extremely small sample size. Asterisks (\*) denote statistically different proportions, Fisher's exact test ( $p = 0.002$ ). Map created using Quantum GIS, version 2.18.

## 3.4 Discussion

### 3.4.1 Presence of *Toxoplasma gondii* oocysts in soil

This study aimed to estimate environmental contamination with *T. gondii* on Phillip Island, which has been proposed as a future release site for the extinct in the wild eastern barred bandicoot.

Estimated cat densities on the island ranged from 1.1 – 4.2 cats/km<sup>2</sup> depending on location, and the prevalence of *T. gondii* infection in the cat population was known to be approximately 80 % (see Chapter 2). Therefore, environmental contamination with oocysts, and by extension exposure risk for eastern barred bandicoots, was expected to be high. However, the present study did not detect *T. gondii* in soil samples from either of the two sites on the island where soil samples were collected. The method used has been estimated to detect oocysts at a concentration of 10-100 oocysts/gram of soil (Lélu et al., 2011). Therefore, the presence of *T. gondii* oocysts at these sites at a lower concentration cannot be excluded.

The absence of *T. gondii* in soil samples in this study contrasts considerably with other, similar studies. For example, in north-eastern France, the proportion of *T. gondii*-positive soil samples ranged from 37.7 – 66.3 %, with known cat densities of 8 - 28 cats/ha (Simon et al., 2017). In another French study, the estimated prevalence of oocysts in soil was 4.8 %, at a cat density of 9.7 +/- 0.5 cats/ha, and a known *T. gondii* seroprevalence of 18.6 % (Afonso et al., 2008). Notably, both studies focused on urban areas or farms, and soil contamination has been shown to decrease with increasing distance from households and farms (Gotteland et al., 2014). The locations in the present study were less inhabited in comparison, although the north-eastern aspect of the Cape Woolamai site is adjacent to a housing estate. Additionally, cat densities were much higher in the French studies when compared to the estimated cat densities on Phillip Island at the time of this study. It may be that despite the high prevalence of *T. gondii* infection in the Phillip Island feral cat population, the lower density of cats resulted in a low level of environmental contamination with oocysts, which was below the level of detection of this study. Moreover, the lower cat density on

Phillip Island may have resulted in a more focal distribution of oocysts i.e. less cats = less and more sparsely distributed defaecation sites, further reducing the likelihood of oocyst detection.

Millions of oocysts may be shed in the faeces of an infected cat, however, the mechanisms by which these oocysts may be distributed further in soil are poorly understood, and therefore the distance they may travel from defaecation sites are difficult to predict. Due to these unknowns, and the effects of variable humidity, temperature, and exposure across different sites, it seems reasonable to assume that oocysts are likely to have a heterogenous distribution in the environment. Oocysts were detected infrequently in soil at randomly selected sites around an urban hospital supporting a cat population (Afonso et al., 2008). However, when sampling was focused on cat latrine sites, as detected by faecal markers, the probability of oocyst detection increased significantly (Afonso et al., 2008). In this study, two suspected feline faecal samples (based on morphology and texture) were identified at Cape Woolamai and processed as per the soil samples, but *T. gondii* DNA was not detected (data not shown). Future studies at Phillip Island could focus sampling around feline faecal deposits or latrine sites, or at known cat locations (e.g. as detected by camera-trapping) to increase the probability of oocyst detection. Since vertical transmission is not considered to be an important mode of transmission of *T. gondii* in cats in nature, free-roaming cats are likely to be first exposed to *T. gondii* at weaning when they begin to eat prey (Afonso et al., 2006; Dubey and Hoover, 1977). As such, weaning age kittens are probably the most significant contributors to environmental contamination with oocysts. In support of this theory, cats living in social groups with kittens are 1.5 times more likely to seroconvert to *T. gondii* with each additional kitten in the group (Afonso et al., 2010). Therefore, high risk sites could be further identified by focusing attentions on locations at which kittens are frequently observed.

The soil at both study sites had a very high sand content due to their coastal location. Some sampling areas were located on dunes, where soil consisted of almost 100 % sand. Lélou et al. (2011)

found that oocyst recovery was much higher in soil with the lowest proportion of sand (30 % sand). They proposed that during the dispersion step (vortexing the sample for 1 minute) fast-moving sand particles may cause damage to oocyst walls, making them permeable to the floatation solution and preventing them from separating into the expected layer after centrifugation. To counter this proposed effect, in this study the dispersion step was reduced to gentle inversion of the tube and vortexing on low speed for 10-15 seconds, or until the sample appeared homogenous. Despite these modifications, it is possible that the large quantities of sand in most samples damaged any *T. gondii* oocysts present and prevented their detection. Future studies could target areas with low sand content; however, due to the nature of the island environment, this would exclude large areas of the island and would therefore limit the usefulness of the sampling.

In this study, a commercial kit designed for extraction of total DNA from faecal samples was used (Bioline, ISOLATE Faecal DNA kit). This contrasts with previous similar studies, which used a kit designed for DNA extraction from tissue or cells, in combination with multiple freeze-thaw cycles (Gotteland et al., 2014; Lélou et al., 2011). PCR-inhibitors are in high quantities in soil, and if not removed during the DNA extraction process, can contribute to false negative results (Schrader et al., 2012). Similar inhibitors are also present in faeces, and hence a faecal DNA extraction kit was chosen for this study as it is designed specifically to remove these compounds. The successful amplification of the EHV internal control in each qPCR reaction showed that PCR-inhibitors were successfully removed from each sample. Though improbable, it is possible that the use of a different DNA extraction technique reduced the likelihood of detection of *T. gondii* DNA in the soil samples in this study. To control for this as best as possible, conventional PCR, using universal eukaryotic primers directed at a conserved region of the 18S rRNA gene, was used to confirm the presence of amplifiable eukaryotic DNA in each soil DNA sample.

### 3.4.2 Prevalence of *Toxoplasma gondii* in rabbits

Rabbits are considered to be relatively resistant to clinical toxoplasmosis, although disease has been reported in domestic individuals (Dubey et al., 1992). Therefore, infected animals are suspected to persist in the population, making rabbits a useful indicator species. In Australia, rabbits are the predominant food source for feral cats (Doherty et al., 2015). Therefore, rabbits are a key element in the *T. gondii* transmission cycle in this environment. Additionally, rabbits occupy a similar ecological niche to eastern barred bandicoots. Although the species have different diets and behaviour, they are terrestrial, of similar size, and both are expected to have significant soil exposure – rabbits via burrowing, and eastern barred bandicoots via foraging in soil for invertebrates. Therefore, it was hypothesised that *T. gondii* exposure in rabbits may approximate expected exposure in eastern barred bandicoots inhabiting the same site.

The overall prevalence of *T. gondii* infection in rabbits collected from Phillip Island was 10.5 % (6.3 - 16.8). To the author's knowledge, this study is the first to estimate *T. gondii* prevalence in feral rabbits in Australia using a PCR-based method. Previous studies have detected seroprevalence ranging from 0 – 26 % across a variety of Australian habitats (Cox et al., 1981; Jakob-Hoff and Dunsmore, 1983; Munday, 1972).

Interestingly, *T. gondii* was not detected in any rabbits collected from Summerland Peninsula. This is consistent with the absence of *T. gondii* DNA in soil samples from the same site. This suggests that environmental contamination with oocysts in this region is very low. Unfortunately, as rabbits were collected opportunistically through a third party, it was not possible to focus rabbit collection at the same locations as the soil study. Thus, prevalence of *T. gondii* infection in rabbits at Cape Woolamai (the other soil collection site) is not known. Conversely, no soil samples were collected from Cape Kitchen, where the only *T. gondii* positive rabbits with known location were collected. Soil samples were not collected from Cape Kitchen, as it was not known that a large proportion of the rabbit

samples would be taken from this site until after the soil samples were collected. Cat density was not known at Cape Kitchen; however, consistently high rabbit populations are suspected to support a higher than normal population of cats (S. Murphy, personal communication). The large cat and rabbit populations are likely to support endemic transmission cycles of *T. gondii* through predation and environmental contamination. Future soil studies should focus on collection from the Cape Kitchen site, because environmental contamination with oocysts is likely to be significantly higher at this location. Interestingly, vertical transmission of *T. gondii* has been demonstrated in rabbits (Uhlíková and Hübner, 1973). Thus, vertical transmission may also be contributing to the high prevalence of *T. gondii* infection in rabbits at Cape Kitchen. Further research is required to determine the significance of this mode of transmission in rabbits at this location.

A single oocyst can be sufficient to establish infection in an intermediate host (Dubey, 2006). In comparison, using currently available methods, oocysts may only be detected at concentrations of 10-100 oocysts/gram of soil (Lélu et al., 2011). Thus, indicator species are likely to be a more sensitive method of detecting environmental contamination with *T. gondii*. This is consistent with the results observed in this study, where *T. gondii* was not detected in soil samples but was detected in rabbits from nearby sites. In the case of Phillip Island, use of indicator species such as rabbits may serve as a better measure for environmental contamination than direct detection of oocysts in soil.

### 3.5 Conclusion

Oocysts were not detected in any soil samples collected from Phillip Island, and therefore it can be assumed that either:

- i) soil samples were truly negative with an estimated prevalence of between 0-2.5 % and 0 - 6.4 % in the Summerland Peninsula and Cape Woolamai, respectively;
- ii) oocyst contamination on the island is below the level of detection of the test used in this study (10-100 oocysts/gram of soil); or,

iii) oocyst distribution is heterogenous and the sampling design did not provide adequate coverage of the study sites to account for this heterogeneity.

Some limitations of this technique, e.g. reduced recovery rates in sandy soil, may have reduced the sensitivity further at the sites surveyed in this study. *Toxoplasma gondii* infection was detected in rabbits from the island, suggesting that there is at least some level of environmental contamination with oocysts. All rabbits sampled from the Summerland Peninsula were negative for *T. gondii* infection, which suggests this location may pose a low toxoplasmosis risk to eastern barred bandicoots released there in the future. Further studies are required to accurately quantify exposure risk and gain deeper understanding of the epidemiology of *T. gondii* on Phillip Island.

## 4 Longitudinal study of eastern barred bandicoots released onto Phillip Island

### 4.1 Introduction

The eastern barred bandicoot (*Perameles gunnii*), mainland subspecies, is a small, omnivorous marsupial native to south-western Victoria and far south-eastern South Australia (Seebeck et al., 1990). The eastern barred bandicoot is currently considered to be 'extinct in the wild', and is the focus of a long-standing species recovery program (Parrott et al., 2017). The species is currently extant only in intensively managed 'reintroduced' populations, including Hamilton Community Parklands, Woodlands Historic Park, and Churchill Island, as well as the captive breeding program, managed by Zoos Victoria (Parrott et al., 2017). These reintroduced populations are free-ranging, but contained within predator-proof fencing, and are routinely supplemented with captive-bred animals. Eastern barred bandicoots are known to be susceptible to toxoplasmosis, the disease caused by the protozoan parasite, *Toxoplasma gondii* (Bettioli et al., 2000a). Toxoplasmosis is a significant disease of this species in captivity, and is a recognised cause of death in wild populations (Lenghaus et al., 1990; Lynch, 2008).

During a recent assisted colonisation trial on French Island, Victoria, four of 18 bandicoots became infected with *T. gondii* following their release (Groenewegen et al., 2017). Disseminated toxoplasmosis was the primary cause of death in one of these animals. A second bandicoot also had disseminated toxoplasmosis at post mortem but had a significant concurrent disease process; it is not known if the disseminated toxoplasmosis was as a result of primary or recrudescent infection (M. Lynch, unpublished data). A third animal had a necrotic focus in the pancreas associated with protozoa, these were assumed to be *T. gondii* but this could not be confirmed as the carcass was significantly autolysed. The final bandicoot seroconverted to *T. gondii* but was lost to follow up; how long this animal survived for is therefore unknown (Groenewegen et al., 2017). The results of this

trial, coupled with historical evidence, suggest that *T. gondii* is a significant threat to the establishment and long-term survival of eastern barred bandicoot populations. However, clinically normal seropositive individuals have also been identified, both in captivity and in the wild, suggesting that *T. gondii* infection may not be universally fatal in this species (Miller et al., 2000; Obendorf et al., 1996). Obendorf et al. (1996) did not re-trap any seropositive individuals over a period greater than three months, and suggested that *T. gondii* infected individuals do not survive long term, even if they can survive acute infection. Thus, chronic infection with *T. gondii* may reduce longevity of eastern barred bandicoots. Subtle behavioural changes (e.g. increased diurnal activity and uncharacteristic docility) that may be caused by chronic infection may reduce fitness in the wild and increase the likelihood of other causes of death, such as predation and motor vehicle accidents (Lenghaus et al., 1990; Obendorf et al., 1996). Therefore, although toxoplasmosis appears to be a significant disease of eastern barred bandicoots, greater understanding of the epidemiology is required, particularly regarding long-term survival of free-ranging chronically infected individuals. Unfortunately, there is a paucity of robust, longitudinal studies assessing the impact of *T. gondii* on wild marsupial populations in general (Hillman et al., 2016).

The present study focused on a population of eastern barred bandicoots translocated to the Summerland Peninsula, Phillip Island, in an assisted colonisation trial. This area is known to be inhabited by feral cats, which have a high prevalence of *T. gondii* infection (this thesis, Chapter 2). Bandicoots were tested for exposure to *T. gondii* using the modified agglutination test (MAT) prior to translocation and then again on two occasions after translocation. The specific aim of this study was to measure morbidity, mortality and *T. gondii* exposure in bandicoots, to gain a greater understanding of the epidemiology of this parasite in a free-ranging population. Additionally, this study sought to investigate the risk toxoplasmosis poses to the establishment and survival of eastern barred bandicoot populations.

## 4.2 Materials and Methods

### 4.2.1 Ethics approval and permits

This work was carried out with the approval of the Phillip Island Nature Parks Animal Ethics Committee, project identification number 22017, and under Wildlife Research Permit: 10008375, Department of Environment, Water, Land, and Planning, Victoria.

### 4.2.2 Trapping, translocation and anaesthesia of eastern barred bandicoots

Eastern barred bandicoots were released onto the Summerland Peninsula, Phillip Island. The Summerland Peninsula is located at the western extremity of Phillip Island and is approximately 424 ha in size. Much of the area is a reclaimed housing estate, and the environment consists predominantly of revegetated *Poa* spp. grassland and *Melaleuca* spp. woodland. Animals were translocated from five locations: Woodlands Historic Park, Churchill Island, Hamilton Community Parklands, and the captive breeding population. Captive-bred animals were sourced from Werribee Open Range Zoo (WORZ), and Serendip Sanctuary. At Woodlands, Hamilton and Churchill, traps were set the evening prior to translocation. Covered wire cage traps and peanut butter, honey and oat baits were used, as reported previously (Winnard et al., 2013b). On Churchill Island, traps were checked during the night and animals were transferred to shredded-paper lined animal transport boxes, provided with food and held in a dark, quiet room overnight. At Woodlands and Hamilton, traps were checked early in the morning, and bandicoots were transported directly to a central, on-site processing station. The morning after capture, animals were anaesthetised with isoflurane via mask for sample collection. Captive animals, including animals from Serendip, were anaesthetised for blood sample collection up to one month prior to translocation at the WORZ veterinary department. In the weeks prior to release, all captive-bred animals underwent a routine transition program, including changing to a modified 'wild' diet, to ensure successful acclimatisation to a free-ranging life style.

During anaesthesia, all bandicoots underwent a physical examination by an experienced wildlife veterinarian. A blood sample was taken from the femoral vein, and collected into serum gel separator, EDTA, and micro-haematocrit tubes. Samples were either centrifuged immediately, or stored, chilled, for a maximum of 12 hr. Separated serum was stored at -20 °C prior to analysis. Packed cell volume (PCV) and total plasma protein (TPP) was measured routinely. Weight, sex, and other demographic data was recorded for each animal. Any animals not already microchipped had a microchip (Trovan, UK) placed subcutaneously in the interscapular region during this procedure. Females with large pouch young were not anaesthetised and did not have a blood sample collected, due to the potential impact of handling and anaesthesia on the pouch young and the increased likelihood of ejection. All bandicoots were treated for endo- and ectoparasites with subcutaneous moxidectin (Cydectin®; Virbac, Carros, France). Captive animals were treated twice, two weeks apart leading up to the release date, and free-ranging animals received a single treatment at the time of anaesthesia. The presence or absence of ectoparasites and type of ectoparasites present was recorded for each animal.

After anaesthetic recovery, animals from Churchill Island, Woodlands and Hamilton were transported in shredded-paper lined animal transport boxes in an air-conditioned vehicle to the release site. Bandicoots from WORZ and Serendip were captured from their enclosures on the day of the release and transported to the release site in the same manner. All bandicoots were released after dusk and monitored for a short period to ensure normal behaviour. Post-release monitoring trapping sessions were carried out twice, at approximately three weeks post-release (mid-November 2017), and approximately 16 weeks post-release (mid-February 2018). Trapping was carried out using traps and baits as described above, using 53 traps set out in a random-origin grid over the release area. The same grid was used in each trapping session. Traps were set for three subsequent nights in each session, totalling 159 trap-nights per session. Traps were opened at dusk and checked from 1-2 hours after sunset until all traps had been checked. Bandicoots trapped during these

sessions that were deemed suitable for anaesthesia (i.e. apparently healthy and absence of large pouch young) were anaesthetised using isoflurane to facilitate collection of a blood sample, as described above. Weight, sex, and other demographic data was recorded for each animal.

Bandicoots were recovered from anaesthesia in shredded-paper lined animal transport boxes.

Bandicoots were considered to be suitably recovered for release when they were bright, alert and responsive, with no signs of ataxia and a minimum of 1 hour had passed since the anaesthesia. All animals were released at the site they were trapped and observed until they moved off from the site. Animals were only anaesthetised once per trapping session.

#### 4.2.3 Radio transmitter attachment and monitoring

A subset of animals was fitted with a tail mounted radio transmitter with thermistor (PIP Ag393; Biotrack, Wareham, UK), as per the method reported previously by Coetsee et al. (2016).

Transmitters were fitted under manual restraint during anaesthetic recovery for animals undergoing anaesthesia, or manual restraint only for animals which did not undergo anaesthesia. Briefly, a small piece of Fixomull<sup>®</sup> (BSN Medical Australia and New Zealand, Mt Waverly, Victoria, Australia) was wrapped around the base of the tail, the transmitter was then positioned over this layer of tape and wrapped in a second layer of tape. This was to avoid direct contact of the transmitter with the tail and prevent the development of pressure sores. Animals were tracked once during the day (nest site) and once during the night for every 24-hour period until the transmitter fell off or could no longer be located (R. Groenewegen, personal communication). Radio-tracking of the bandicoots was the focus of a separate research project carried out by collaborators, and therefore details are not reported here. The significance of radio-tracking for this study was the ability to locate deceased bandicoots.

#### 4.2.4 Modified agglutination test

The Modified Agglutination Test (MAT) was performed using a commercially available kit (Toxo-Screen DA, bioMerieux, Mercy l'Etoile, France) with minor adaptations, according to the method developed by Mt Pleasant Laboratory, Department of Primary Industries, Parks, Water, and Environment, Tasmania, as described in Chapter 2 of this thesis. Individuals with a titre of  $\geq 1/64$  were considered *T. gondii* seropositive, this cut-off value has been previously reported for eastern barred bandicoots (Miller et al., 2000; Obendorf et al., 1996). The same observer performed all tests.

#### 4.2.5 Recovery of deceased eastern barred bandicoots and necropsy protocol

Deceased bandicoots that were recovered underwent a thorough necropsy to determine the cause of death. If immediate necropsy was not possible, carcasses were stored at -20 °C until processing. Representative samples of brain, heart, lung, liver, spleen, kidney, and skeletal muscle were collected in 4% formalin for histopathology. The remainder of each organ (or approximately 6 g for skeletal muscle) was collected and stored at -20 °C. Total DNA was extracted from each organ sample, and each sample was tested for the presence of *T. gondii* DNA by qPCR according to the protocols described in detail in Chapter 2 of this thesis.

#### 4.2.6 Statistical analyses

Statistical analyses were carried out using Excel 2016 (Microsoft, Redmond, Washington, USA), Minitab<sup>®</sup>, version 18.1 (Minitab Inc, State College, Pennsylvania, USA), and Stata (StataCorp, College Station, Texas, USA). Paired t-tests for dependent samples were used to compare mean body weight, PCV, and TPP of bandicoots across each different sampling period and any unpaired samples were excluded from these statistical analyses. Two-tailed Fisher's exact tests were used to compare sample proportions when samples were independent (i.e. proportion of males and females re-trapped at least once). Two sample t-tests were used to compare independent sample means (e.g. mean PCV of male and female bandicoots prior to release). Mixed model logistic regression was used

to compare likelihood of ectoparasite infestations across each trapping period. Population estimates for disease freedom calculations were determined using open population (robust design) mark-recapture models, allowing for births and deaths, but constrained to limited emigration (Table 4.1) (D. Sutherland, unpublished data). The models were not spatially explicit. Population numbers provided were considered to be an estimate of the total number of adult eastern barred bandicoots present on the trapping grid during each session. Bandicoots that had dispersed off the grid area entirely were not included in the estimate. Calculations for disease freedom were made using “FreeCalc” from EpiTools epidemiological calculators (Sergeant, 2018). The modified hypergeometric exact method for small populations was used. Specified design prevalence was 6%, based on previous studies of seroprevalence in free-ranging eastern barred bandicoots (Obendorf et al., 1996). The null hypothesis was that the disease was present in the population at greater than or equal to the specified design prevalence. Test specificity was estimated as 93% based on the specificity of the MAT in a closely related species, the southern brown bandicoot (*Isodon obesulus*), as calculated by Hillman et al. (2017). The calculation was run with three different estimates for test sensitivity, 70%, 80% and 90%.

Table 4.1: Population estimates for eastern barred bandicoots on Summerland Peninsula, Phillip Island during different trapping sessions based on mark-recapture modelling (D. Sutherland, unpublished data).

<b>Trapping Session</b>	<b>Population Estimate (95% confidence interval)</b>
<b>November 2017</b>	44 (38 – 59)
<b>February 2018</b>	40 (35 – 55)

## 4.3 Results

### 4.3.1 Translocation of eastern barred bandicoots to the Summerland Peninsula

In October 2017, 44 eastern barred bandicoots were translocated to the Summerland Peninsula, Phillip Island, from Woodlands Historic Park (10 animals), Churchill Island (23 animals), WORZ (9 animals), and Serendip Sanctuary (2 animals). A further 17 animals from Churchill Island were translocated in early November. Six animals from Hamilton Community Parklands were translocated in early December. In total, 67 animals were released, 35 males and 32 females. Three females from Woodlands, and three females from Churchill Island did not have a blood sample collected prior to release due to the presence of large pouch young. Overall, 17 females with pouch young were translocated. In total, 61 of the 67 bandicoots were sampled prior to release on Summerland Peninsula.

### 4.3.2 Re-trapping of bandicoots after translocation to the Summerland Peninsula

In November 2017, approximately 3 weeks after the initial release, 27 bandicoots (44.3% of total number bandicoots released on the peninsula by November, Table 4.2) were re-trapped. Three of the re-trapped bandicoots had been translocated in the second group in early November. These animals had been recently anaesthetised for a pre-release blood sample and had not been present on the peninsula long enough to develop an antibody response, therefore no blood sample was collected from these animals. Nine females had pouch young; one of these had very large pouch young and therefore was not anaesthetised for blood collection. As a result, blood was collected from 23 bandicoots in November, representing 37.7% of the total known population at that time (Table 4.2). In February 2018, approximately 16 weeks post-release, 31 bandicoots (46.3% of total number of bandicoots released on peninsula, Table 4.2) were re-trapped. One 'cleanskin' (i.e. no microchip present, either translocated as pouch young or born on the peninsula) was also trapped in February. Twelve females had pouch young in February, and 11 of these were not anaesthetised for

blood collection due to the very large size of the pouch young. In total, blood samples were collected from 21 bandicoots in February, representing 31.4% of the total known population at that time (Table 4.2). Overall, 59.7% of released individuals (n=40) were re-trapped at least once over the two trapping periods; 26.8% (n=18) were re-trapped in both sessions. The total number of individuals re-trapped at least once over the entire study period was 21 (65.6%) for females, and 19 (54.3%) for males. There was no significant association between sex and likelihood of being re-trapped (difference = 0.11, 95% CI -0.11 – 0.35; p = 0.455, two-tailed Fisher’s exact test).

Table 4.2: Number of eastern barred bandicoots trapped and sampled (in relation to total number of animals released) on Summerland Peninsula in November 2017 and February 2018 trapping sessions.

	<b>Total number trapped (% of total released)</b>	<b>Number sampled (% of total released)</b>	<b>Total number released by time of sampling</b>
<b>Pre-release</b>	67 (100)	61 (91.0)	N/A
<b>November</b>	27 (44.3)	23 (37.7)	61
<b>February</b>	31 (46.3) *	21 (31.4)	67

\* One cleanskin (i.e. born on the peninsula) bandicoot was also trapped during February, and therefore the total number caught overall during this session was 32.

#### 4.3.3 Body weight of bandicoots prior to release and after translocation to the Summerland Peninsula

The mean body weights of male and female bandicoots prior to release were 747 grams (95% confidence interval [CI] 717 – 777) and 647 g (605 – 690), respectively (Figure 4.1). Prior to release, males were significantly heavier than females (difference = 99 g, 95% CI 49 – 151; p < 0.001, two sample t-test). There was no significant difference between the weight of male and female

bandicoots in November ( $p = 0.141$ ), or February ( $p = 0.059$ ). However, there was a trend for males to be heavier than females on both occasions (Figure 4.1). Therefore, for further analyses of weight change, the data was stratified by sex. The mean body weights of male bandicoots during the November and February trapping sessions were 774 g (749 - 799) and 827 g (750 - 904), respectively (Figure 4.1). Comparison of the mean body weight of male bandicoots pre-release and in November showed a statistically significant difference, with a mean weight gain of 29 g (95% CI 12 - 46;  $p = 0.002$ , paired t-test) (Figure 4.2). Between November and February, males had a mean body weight gain of 88 g (60 - 115), which was statistically significant ( $p < 0.001$ ). Over the course of the study, i.e. pre-release to February, there was a mean body weight gain of 125 g (107 - 144) in male bandicoots, which was statistically significant ( $p < 0.001$ ). The mean body weight of female bandicoots in November was 723 g (655 - 791) which increased to 741 g (689 - 793) in February (Figure 4.1). There was a mean body weight gain of 83 g (26 - 140) in female bandicoots between pre-release and November, which was statistically significant ( $p = 0.009$ , paired t-test) (Figure 4.2). Between November and February, female bandicoots had an average weight gain of 61 g (26 - 95), which was statistically significant ( $p = 0.003$ ). Over the course of the study, i.e. pre-release to February, there was a mean body weight gain of 100 g (53 - 147) in female bandicoots, which was statistically significant ( $p < 0.001$ ). There was no significant difference in absolute weight gain between male and female bandicoots over the course of the study (difference = 25 g, 95% CI -24 - 75;  $p = 0.305$ , two sample t-test). There was also no significant difference in percentage weight gain between male and female bandicoots over the course of the study (1.5%, 95% CI -8.6 - 11.6;  $p = 0.765$ ). Except for two males and three females, all re-trapped bandicoots increased or maintained body weight over the sampling period. Only one female lost a clinically significant percentage of body weight (8.2%), the other females lost 0.8%, and 3.0%, respectively. The two males lost 3.4% and 2.1% of body weight, respectively.

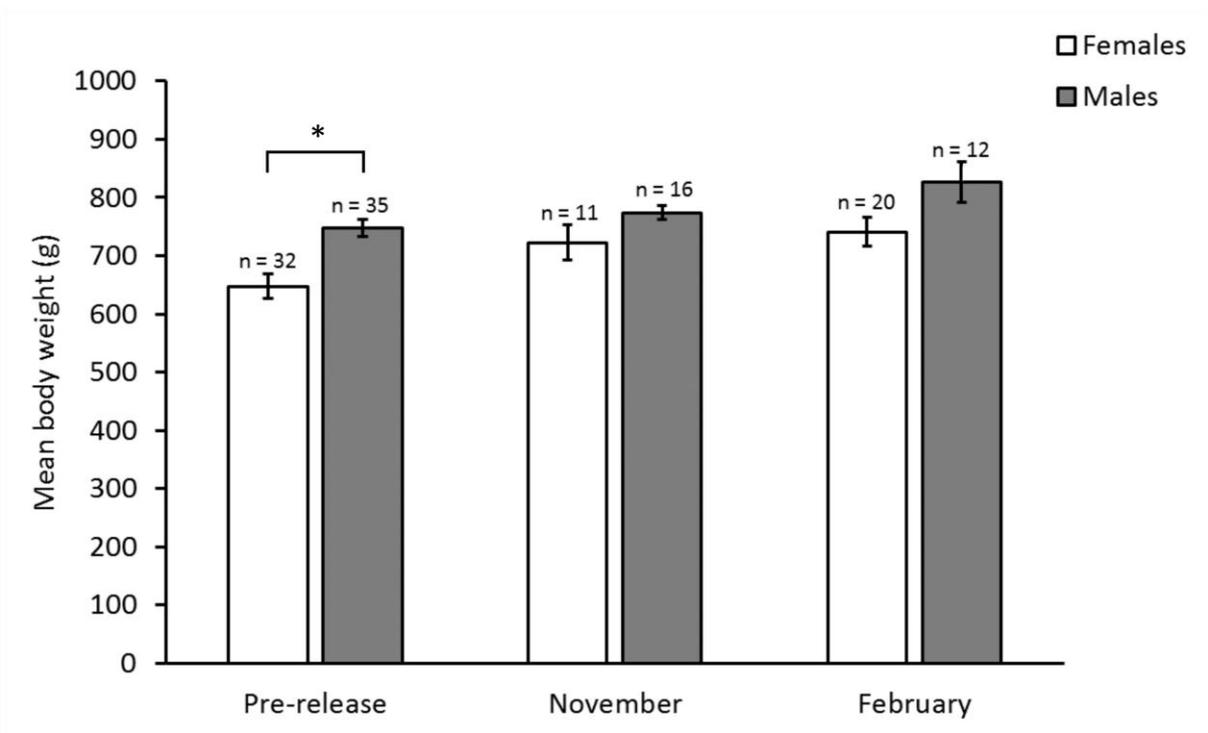
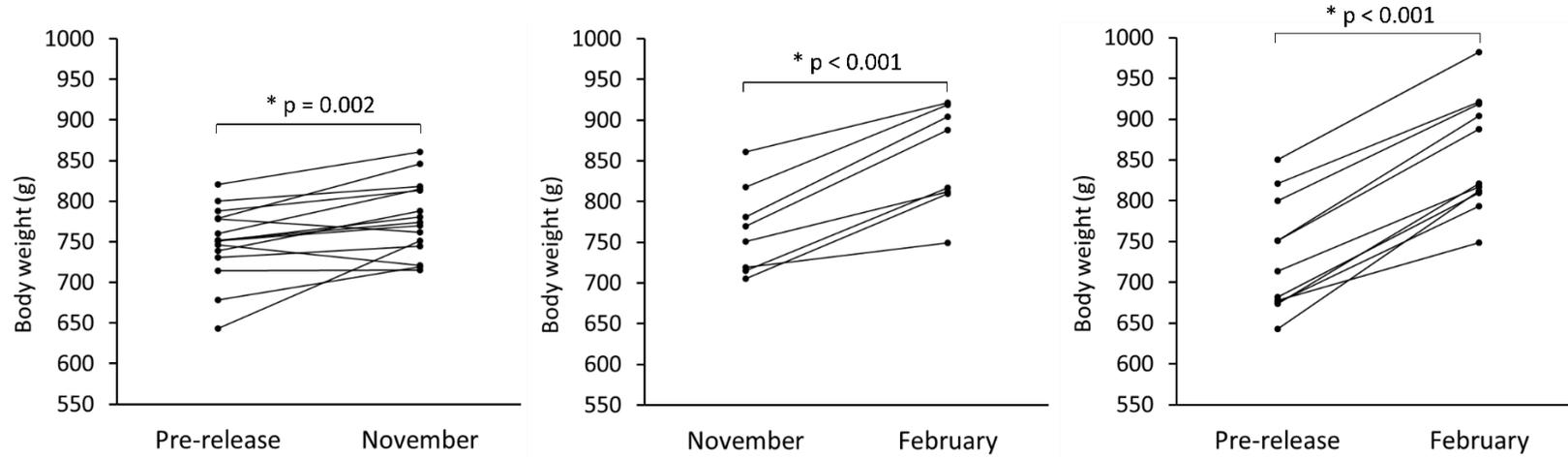


Figure 4.1: Overall mean body weights for male and female eastern barred bandicoots prior to release and in each trapping session on Summerland Peninsula, Phillip Island. Error bars indicate standard error of the mean. Asterisks denote statistically significant differences.

\*  $p < 0.001$ , two sample t-test.

### Males



### Females

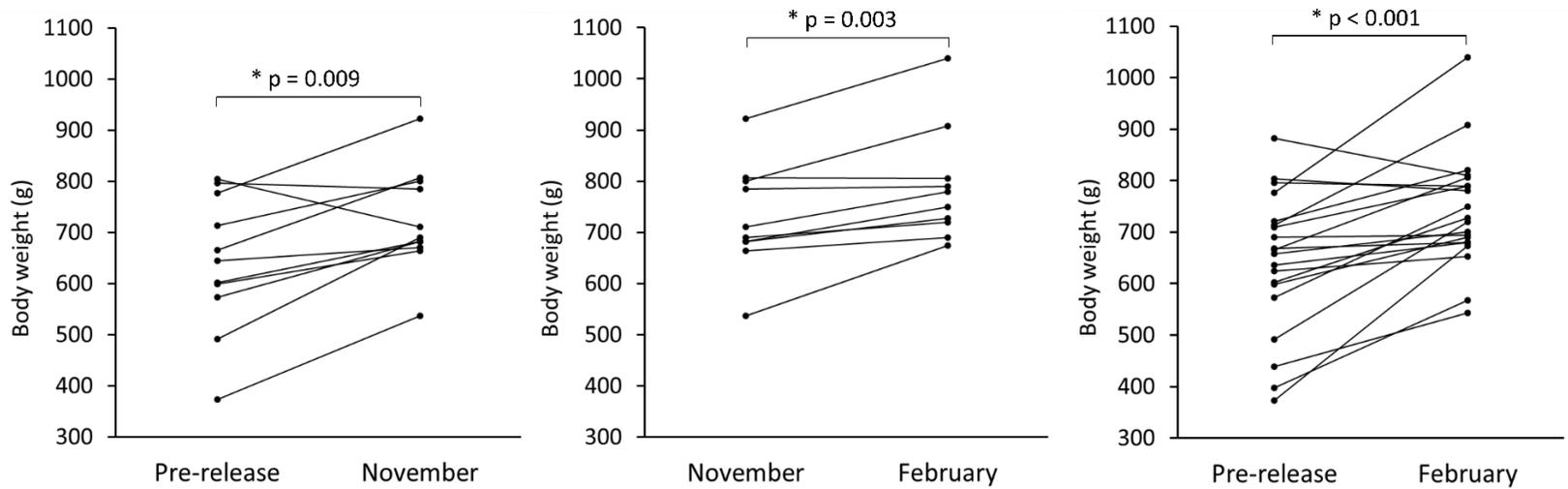


Figure 4.2: Body weight comparisons of male and female eastern barred bandicoots pre-release and in November and February trapping sessions on Summerland Peninsula, Phillip Island. Only paired samples are shown. Asterisks denote statistically significant differences, paired t-test.

#### 4.3.4 Blood parameters (PCV and TPP) of bandicoots prior to release and after translocation to the Summerland Peninsula

The mean PCV of bandicoots prior to release was 44 (95 % CI 43 – 45), compared to 43 (41 – 44) in November and 43 (41 – 45) in February (Figure 4.3 4.3). There was no significant difference in mean PCV between male and female bandicoots pre-release (difference 1.4, 95% CI -0.4 – 3.1;  $p = 0.117$ , two sample t-test), nor in November ( $p = 0.365$ ) or February ( $p = 0.411$ ). Therefore, data was not stratified for sex for comparisons of mean PCV between each trapping session. There was no significant difference between mean PCV pre-release and in November (difference = 1.1, 95% CI -0.6 – 2.8;  $p = 0.203$ , paired t-test), or the mean PCV in February (0.9, -0.8 – 2.6;  $p = 0.275$ ) (Figure 4.4).

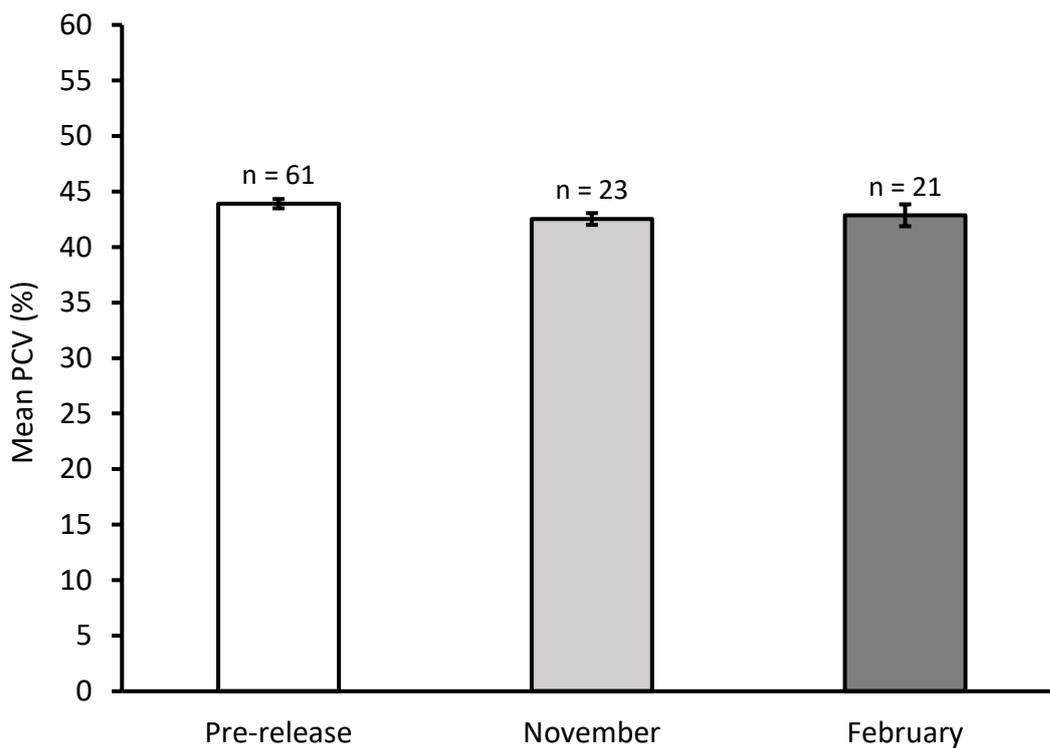


Figure 4.3: Overall mean packed cell volume (PCV) for eastern barred bandicoots prior to release and in each trapping session on Summerland Peninsula, Phillip Island. Error bars indicate standard error of the mean.

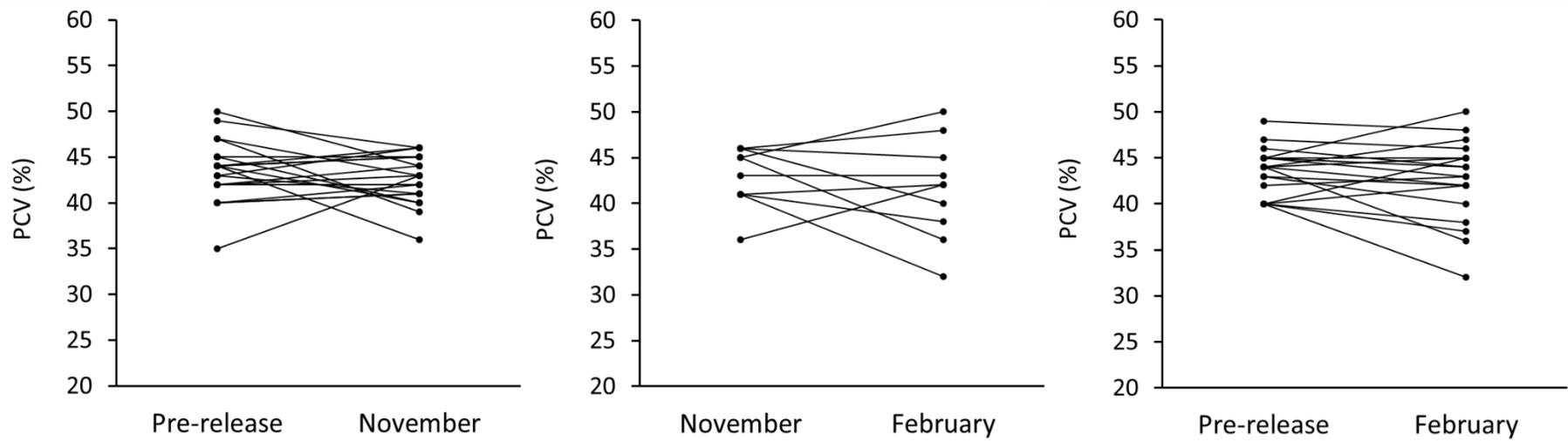


Figure 4.4: Packed cell volume (PCV) comparisons of eastern barred bandicoots pre-release and in November and February trapping sessions on Summerland Peninsula, Phillip Island. Only paired samples are shown.

The overall mean TPP of bandicoots pre-release was 51 g/L (95 % CI 49 – 53), compared to 56 g/L (53 – 58) in November, and 56 g/L (54 – 59) in February (Figure 4.5). Observation of histograms of the TPP data revealed that the data distribution in February was skewed to the right. Therefore, to achieve a normal distribution and satisfy the assumption of the parametric tests, TPP data was log (base 10) transformed for analysis. At release, males had a significantly higher TPP than females ( $p = 0.003$ , two sample t-test) (Figure 4.5). This was no longer true in November ( $p = 0.764$ ) or February ( $p = 0.357$ ). However, there was still a trend for higher TPP in males compared to females in February. Therefore, TPP data was stratified by sex for comparisons between pre-release and each trapping session. There was no significant difference in mean TPP of males measured prior to release compared to November ( $p = 0.099$ , paired t-test), or between November and February ( $p = 0.186$ ) (Figure 4.6). However, the mean TPP of males was higher in February compared to pre-release, and this difference was statistically significant ( $p < 0.001$ ). There was no significant difference in mean TPP for females prior to release when compared to November ( $p = 0.182$ ), or February ( $p = 0.122$ ) (Figure 4.6). There was also no significant difference in mean TPP of females between November and February ( $p = 0.365$ ). Although it was not statistically significant, there was a trend for increasing TPP over the course of the study in females, i.e. from pre-release to February.

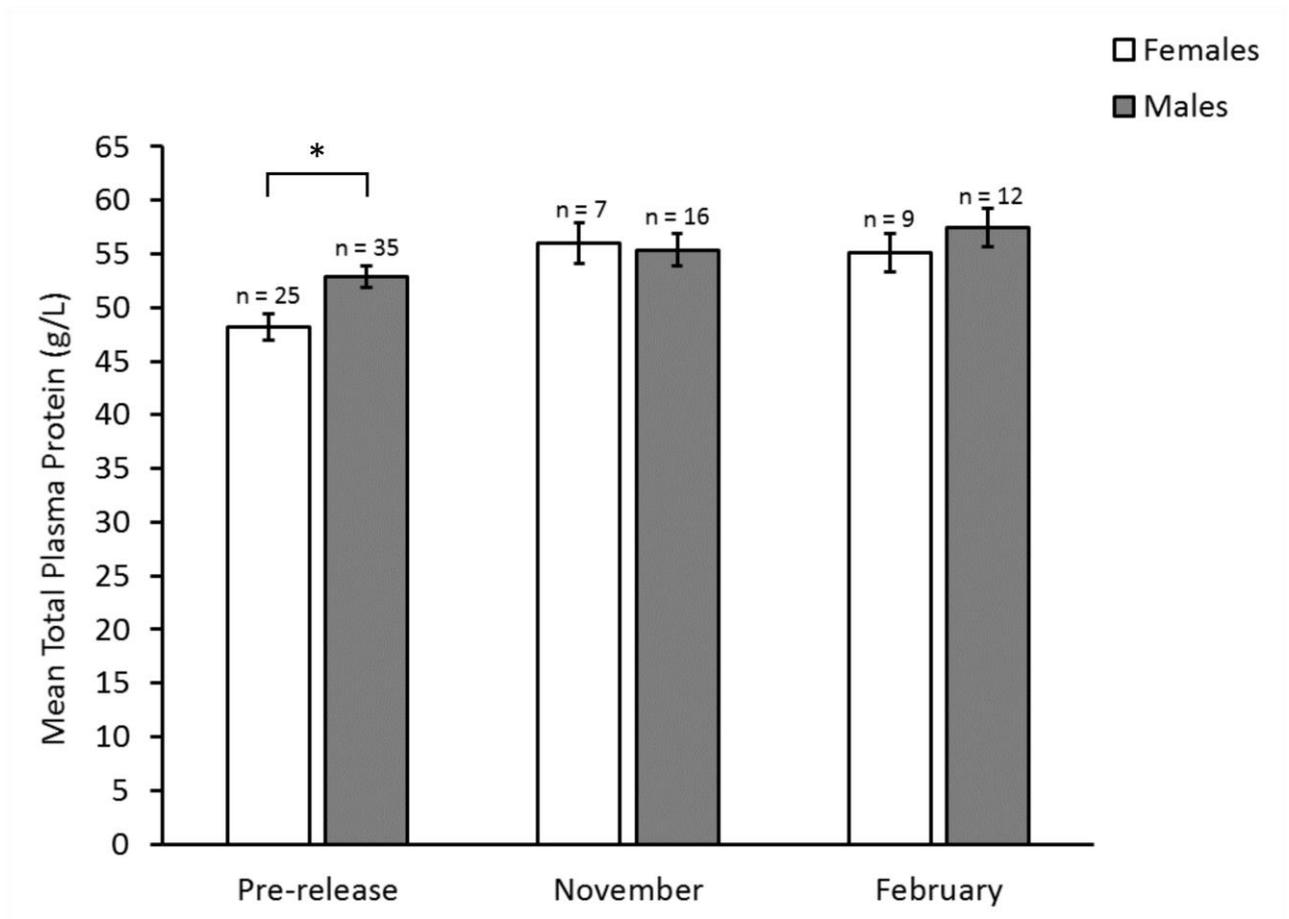
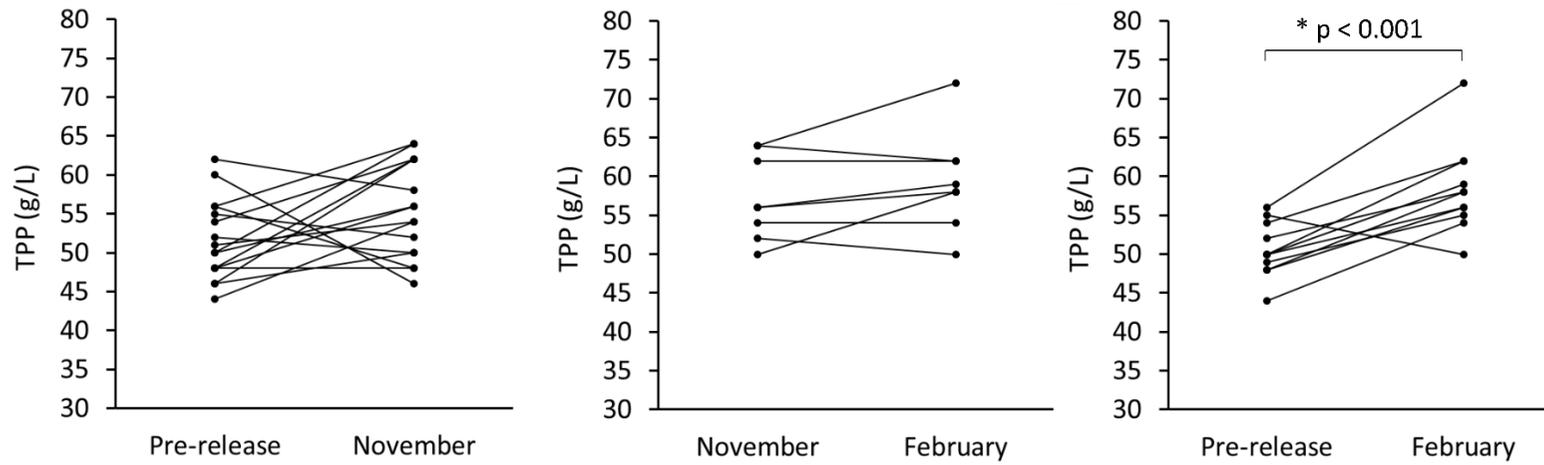


Figure 4.5: Overall mean total plasma protein (TPP) for male and female eastern barred bandicoots prior to release and in each trapping session on Summerland Peninsula, Phillip Island. Error bars indicate standard error of the mean. Asterisks denote statistically significant differences.

\*  $p = 0.003$ , two sample t-test. Two sample t-test performed on  $\log_{10}$  transformed data, but data points are displayed untransformed for ease of interpretation.

### Males



### Females

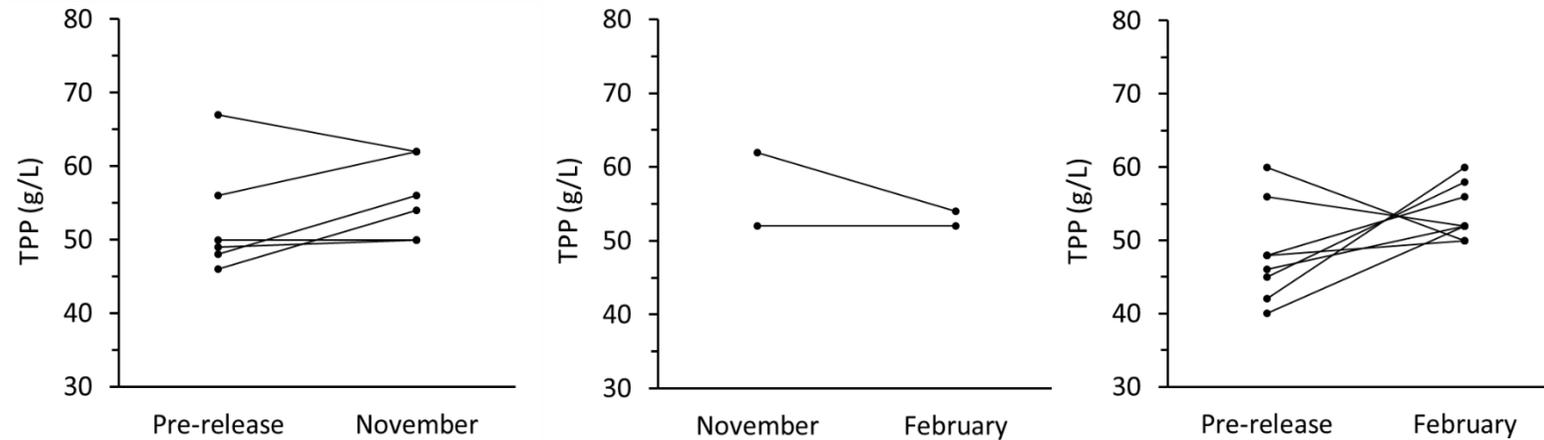


Figure 4.6: Total plasma protein (TPP) comparisons of male and female eastern barred bandicoots pre-release and in November and February trapping sessions on Summerland Peninsula, Phillip Island. Only paired samples are shown. Paired t-tests were performed with log<sub>10</sub> transformed data, but data points are displayed untransformed for ease of interpretation, asterisks denote statistically significant differences.

#### 4.3.5 Ectoparasite infestations of bandicoots prior to release and after translocation to the Summerland Peninsula

Prior to release, 16.4% (95 % CI 8.5 - 27.5) of bandicoots had ectoparasite infestations, consisting of ticks (n = 9 bandicoots) and fleas (n = 3 bandicoots). Ectoparasites were only found on bandicoots from Churchill Island prior to release. These were collected for future species identification. Ticks were predominantly found around the scrotum, axillae, and hind limbs. In November, 37.0% (21.5 – 55.8) of animals had ectoparasite infestations, consisting of ticks (n = 6 bandicoots) and fleas (n = 5 bandicoots). In February 15.6% (6.9 – 31.8) of animals had ectoparasites, consisting of ticks (n = 3 bandicoots) and fleas (n = 2 bandicoots). Mixed model logistic regression analysis was used to assess any association between trapping session and probability of ectoparasite infestation. Bandicoots trapped in November were significantly more likely to be infested with ectoparasites, when compared to pre-release (odds ratio [OR] 2.99, 95% CI 1.09 – 8.25; p = 0.034). There was no significant difference in the probability of ectoparasite infestation prior to release and in February (p = 0.920). There was also no significant difference in the probability of having ectoparasites in November compared to February (3.18, 0.92 – 10.9; p = 0.066). However, there was still a trend suggestive of increased likelihood of ectoparasites in November as indicated by the strong skew of the confidence interval. A greater sample size may have revealed a statistically significant effect.

#### 4.3.6 Exposure to *Toxoplasma gondii* in bandicoots

All bandicoots tested (61 animals pre-release, 23 animals in November, and 21 animals in February) were negative on the modified agglutination test. The probability of the null hypothesis in pre-release animals was < 0.001, when test sensitivity was assumed to be 90%. In other words, the probability of observing  $\leq 0$  reactors in a sample of 61 individuals from a population (N = 67) with a disease prevalence of 6% was <0.001. This was adequate to reject

the null hypothesis and conclude that the population was free from disease (Table 4.3). The probability of the null hypothesis in November was 0.027, when test sensitivity was estimated at 90%. This was sufficient to reject the null hypothesis and conclude that the population was free from disease (Table 4.3). The probability of the null hypothesis in February was 0.06. This was inadequate to reject the null hypothesis. Therefore, in February, the population could not be concluded to be free from disease although no test-positive animals were detected. These calculations were repeated with test sensitivity estimates of 80% and 70%, and consistent results were obtained (data not shown).

Table 4.3: Results of modified agglutination testing (MAT) of Summerland Peninsula eastern barred bandicoots for *Toxoplasma gondii*. Disease freedom status pre-release, and during November and February trapping sessions are indicated, based on assumed test sensitivity of 90% and a design prevalence of 6%.

	<b>Population size</b>	<b>Number tested (positive)</b>	<b>Probability of null hypothesis</b>	<b>Population free from disease?</b>
<b>Pre-release</b>	67 (known)	61 (0)	< 0.001	Yes
<b>November 2017</b>	44 (estimate)	23 (0)	0.027	Yes
<b>February 2018</b>	40 (estimate)	21 (0)	0.06	Unable to reject null hypothesis

#### 4.3.7 Cause of death in recovered deceased bandicoots

A single deceased bandicoot was recovered. This animal became entrapped in an out-of-use, underground, concrete tank. Due to the thickness of the tank walls, this animal was difficult to locate by radio-tracking and was not found for several days. The carcass was autolysed and fly-

blown by the time it was recovered, and histopathology was not performed. The assumed cause of death was dehydration +/- starvation due to the entrapment. All tissues examined were negative on *T. gondii* qPCR.

#### 4.4 Discussion

Although the main aim of this study was not to directly assess the success of the assisted colonisation trial of eastern barred bandicoots on Phillip Island (rather it was to investigate the risk of toxoplasmosis at this location), some measures of success can still be determined from the data collected. The criteria used by Groenewegen et al. (2017) to determine the success of an assisted colonisation trial of eastern barred bandicoots were the following:

- 1) Appropriate habitat use,
- 2) Recovery of release body weight, and
- 3) Founder survival exceeding 100 days.

Habitat use was not assessed in this study and will not be discussed further. In contrast to other translocations of this species (Groenewegen et al., 2017), bandicoots on the Summerland Peninsula did not show an initial trend of significant weight loss. It is possible that weight loss occurred initially, and bandicoots had mostly recovered by the November trapping session, which was approximately three weeks after release. However, comparison of paired samples showed a statistically significant increase in body weight in both male (mean increase 29 g) and female (mean increase 83 g) bandicoots by November. This suggests that significant weight loss immediately after release was unlikely. The greater magnitude of weight gain observed in female bandicoots during this period is likely because several juvenile females were released, and weight gain in these individuals corresponded to growth as well as increasing body condition. Further weight gain was observed in both sexes between November and February, and over the course of the study (Figure 4.2). This suggests that there were

ample food sources available for the bandicoots on Summerland Peninsula, and satisfies criteria 2. At least 31 founder bandicoots (46.3%), were known to be alive approximately 16 weeks (~ 115 days) post release (February), satisfying criteria 3. Additionally, 12 females with pouch young were trapped in February. The pouch life of an eastern barred bandicoot is approximately 55 days (Backhouse et al., 1994), and therefore these pouch young were born on the Summerland Peninsula. This further suggests that the peninsula was an excellent site for the assisted colonisation of eastern barred bandicoots.

Packed cell volume and TPP of bandicoots were measured as further indicators of general health. There was no significant difference in PCV between any of the sampling periods (Figure 4.4). The mean PCV during each period was within reported ranges for healthy eastern barred bandicoots (Lynch, 2008). The mean TPP pre-release was within previously reported ranges; however, mean TPP in November and February was slightly higher than these ranges (Lynch, 2008). This may be explained by the fact that the reported range was obtained from captive eastern barred bandicoots, and the November and February samples in this study were taken from free-ranging bandicoots only (although some individuals did have a captive origin). Total plasma protein was significantly higher in males than in females prior to release (Figure 4.5). To the author's knowledge, this has not been reported previously. Physiological differences, altered dietary preferences, or different foraging habits may be responsible for the increased TPP observed in males. Over the course of the study, i.e. pre-release to February, there was a significant increase in TPP in males (Figure 4.6). This trend was also present in females; however, it was not statistically significant. There was no concurrent increase in PCV, which suggests that the increase in TPP was not due to dehydration. The increase in TPP may reflect a change in dietary composition for bandicoots on Summerland Peninsula compared to their origin. The majority of translocated bandicoots (n = 40) were collected from Churchill

Island, which is only ~20km east of the peninsula. However, Churchill Island has largely been cleared for farm land and currently maintains a heritage farm with a variety of domestic livestock species. This may have led to a different invertebrate diversity (i.e. variation in available dietary items) on Churchill Island compared to Summerland Peninsula, and may account for the increased TPP observed in bandicoots after translocation. Additionally, lipemia, which was assumed to be post-prandial, was observed in some samples collected from bandicoots from the Summerland Peninsula in November and February and this may have falsely elevated the refractometer TPP readings (George, 2001). Regardless of the cause of the increase in TPP in male bandicoots over the course of the study, the absolute increase was small (~ 5 g/L) and is unlikely to have any clinical significance for the bandicoots.

Free-ranging bandicoots sampled in this study were found to have ectoparasite infestations consisting of ticks and fleas. This is a normal expected occurrence in wild populations, and while severe infestations can be associated with disease, there was no indication that the presence of these parasites was adversely affecting the individuals in the present study (e.g. poor body condition, anaemia) (Lenghaus et al., 1990). Bandicoots were more likely to be infested with ectoparasites in November than they were prior to release. This is likely reflective of two things. Firstly, bandicoots sampled prior to release included a group of captive bandicoots (n = 11, 16.4%) which were maintained under quarantine conditions and routinely treated for parasites; none of these animals were affected. Secondly, increasing ectoparasite levels in November were also likely to be influenced by seasonality of the parasites. Although the species was not identified, ticks were likely to be *Ixodes tasmani*, the 'common marsupial tick', as this parasite is frequently found on eastern barred bandicoots (Lenghaus et al., 1990). Studies have shown that *I. tasmani* is found with increasing prevalence in spring on other marsupial hosts, such as brushtail possums (*Trichosurus vulpecula*)

(Murdoch and Spratt, 2006). Ticks and fleas were collected for species identification which will form a part of future research.

No bandicoots were identified as seropositive to *T. gondii* over the course of the study. Prior to release, and in the November trapping session, it could be concluded that the population was free from *T. gondii*, at a design prevalence of 6% (Table 4.3). It was not possible to conclude that the population was free from *T. gondii* infection in February, due to the smaller sample size. The limitation of this calculation is that it is possible for the population to be concluded to be free from disease when a small number (i.e. < 6%) of infected animals are present. Prior to release, the population could be concluded to be free from disease at a design prevalence as low as 2% (or 1 infected individual), however, this was not possible in November due to the smaller sample size (data not shown).

Due to the nature of working with free-ranging wildlife, it was not possible to re-sample each individual bandicoot released onto Summerland Peninsula at each time point. Recapture rates were excellent, with 44.3% of all released animals trapped in November, 46.3% trapped in February, and 59.7% of released individuals (n=40) re-trapped at least once. However, this also means that approximately 40% of bandicoots were not re-captured after release onto the peninsula. It is possible that some of these individuals were exposed to and seroconverted to *T. gondii*. There are several reasons why some individuals may not have been re-trapped. The most obvious are trap avoidance and death; however, dispersal off the trapping grid is also very likely. Traps were required to be set in the same grid locations each time to allow for the calculation of population estimates for each trapping session, using the open population mark-recapture model (D. Sutherland, unpublished data). The peninsula is bordered by a perimeter road, but there are no fences preventing dispersal of animals throughout this area. This

includes eastward to the greater part of the island, although the presence of a large building and car park (Phillip Island Nature Parks “Penguin Parade” building), which is an area of significant human activity, likely discourages dispersal in this direction. Additionally, vehicle access to the peninsula is ceased from sunset to sunrise (when the bandicoots are active), and therefore the road likely does not represent a significant barrier to dispersal. Future research will involve trapping over a wider area to detect dispersed individuals, and measure how far they have moved from their original release site.

A major limitation of this study is the lack of robust mortality data for bandicoots after translocation onto the Summerland Peninsula. The initial aim for this study was to use radio-tracking collars to enable long-term monitoring of the animals, which would have also allowed for accurate identification and collection of any deceased animals. However, due to the difficulties in designing such a product to suit the anatomy of eastern barred bandicoots, an appropriate collar design was not achieved before the commencement of this research. Therefore, tail-mounted transmitters, fitted with a thermistor to act as a mortality indicator, were deployed. These transmitters have a limited attachment time, with a mean of 19 days and a range of 7-33 days (Coetsee et al., 2016). This limited the potential recovery of deceased bandicoots. One deceased bandicoot was recovered in the first week post-release. This animal’s death can be attributed to misadventure and resultant starvation. It was not found to have any evidence of *T. gondii* infection when tissues were tested by qPCR. Although no other deceased bandicoots were recovered, the possibility that bandicoots were becoming infected with *T. gondii* and dying of fatal toxoplasmosis cannot be excluded. If these individuals quickly succumbed to fatal disease, this could explain why no *T. gondii* seropositive bandicoots were detected in this study. More frequent trapping and sampling of individuals (e.g. monthly), may identify seropositive individuals before they succumb to disease. However, in an experimental

infection trial in two eastern barred bandicoots, both animals had a negative MAT titre at the time of death due to toxoplasmosis (Bettioli et al., 2000a). This indicates that some exposed animals, particularly those exposed to a high infectious dose, may be missed regardless of the frequency of sampling.

#### 4.5 Conclusion

Exposure to *T. gondii* was not detected by MAT in blood samples taken from bandicoots at 3 weeks or 16 weeks after release onto the Summerland Peninsula, an environment inhabited by feral cats with a high prevalence of *T. gondii* infection. Recapture rates of bandicoots suggested excellent survivability, and measurement of general health parameters, including body weight, PCV, and TPP suggested that bandicoots have adapted well to the peninsula. However, due to the inability to monitor animals with long-term tracking devices, robust mortality data was lacking in this study. Although no live bandicoots infected with *T. gondii* were detected, death of bandicoots due to toxoplasmosis on the peninsula could not be excluded. Future research should be directed at developing the means to reliably detect deceased animals, so that they can be submitted for necropsy and testing to determine if *T. gondii* has a role as a cause of mortality in this population.

## 5 General Discussion

### 5.1 Revisiting the aims of this thesis

The success of the species recovery of the eastern barred bandicoot, currently classified as extinct in the wild, is reliant on the development of a self-sustaining population. Due to the decimation of the native habitat of this species, and the presence of (and difficulty controlling) introduced predators across its historic range, the establishment of new populations hinges on the identification of 'safe' habitat. Phillip Island was proposed as a site for an assisted colonisation trial for eastern barred bandicoots, due to the presence of large areas of suitable habitat. However, the island is known to have a feral cat population. Eastern barred bandicoots are susceptible to toxoplasmosis, and this was a significant cause of mortality in an assisted colonisation trial on neighbouring French Island (Groenewegen et al., 2017). Therefore, this study aimed to investigate the risk toxoplasmosis poses to the establishment of eastern barred bandicoots in suitable habitat, but in the presence of feral cats.

The aims of this study were broadly to:

1. Establish prevalence of *T. gondii* infection in feral cats on Phillip Island and define risk factors associated with infection.
2. Investigate environmental contamination with *T. gondii* directly by detecting oocysts in soil, and indirectly using feral rabbits as an indicator species.
3. Investigate exposure to *T. gondii* and its role in mortality of eastern barred bandicoots released onto Phillip Island in an assisted colonisation trial.

## 5.2 Overview of major findings

The prevalence and seroprevalence of *T. gondii* infection in the feral cat population on Phillip Island were found to be extremely high, at 79.5% and 91.8%, respectively. This is consistent with other studies investigating the prevalence of *T. gondii* infection in cats on Australian islands (Adams et al., 2008; Fancourt and Jackson, 2014; O’Callaghan et al., 2005). To the author’s knowledge, this is the first study to report the prevalence of *T. gondii* infection in feral cats in Australia using qPCR to detect bradyzoites in the major tissue predilection sites in this host. Bayesian analysis indicated that the specificity of the *T. gondii* qPCR method used in this study was very high (96.2%). In comparison, the sensitivity, while still being diagnostically useful, was lower (90.1%). The sensitivity of the MAT was very high (96.1%); however, the specificity of this test was relatively low (82.0%). Therefore, use of the MAT in epidemiological studies in cats should be interpreted with caution, as false positives may be likely. For future surveillance to be conducted in the most cost, time, and labour-efficient manner, studies could be designed to use the MAT and qPCR in series. As the MAT has a very high sensitivity, the negative predictive value of this test is high. Additionally, the MAT is cheaper and allows a faster throughput of samples than the qPCR. Therefore, initial screening could be conducted with the MAT. Any MAT positives should then be confirmed as *T. gondii* infected with qPCR. The limitation of this approach is that serum and tissue samples would be required from each animal.

*Toxoplasma gondii* infection in cats was positively associated with weight. Furthermore, risk of infection was found to be associated with season, with increased risk of infection in cats trapped in winter and spring. This finding corroborates with other studies from Europe (e.g. Afonso et al., 2013) and has significant implications for the relative risk of *T. gondii* infection in intermediate hosts. Increasing infection risk for cats suggests a preceding increase in infection

risk for intermediate hosts, as cats are most likely to be infected via the carnivorous pathway (Dubey, 2006). Therefore, for those intermediate host species that are susceptible to toxoplasmosis, the highest risk periods on Phillip Island are likely to be autumn, winter, and spring.

Despite the very high prevalence of infection in the feral cat population, *T. gondii* oocysts could not be detected in any soil sampled from Phillip Island. While this may represent a very low level of oocyst contamination at the sites tested, it is also possible that the sensitivity of the technique and/or study design was not sufficient to detect oocysts at these locations. *Toxoplasma gondii* infection was only identified in feral rabbits from Phillip Island from one site, Cape Kitchen. No infection was detected in rabbits from the Summerland Peninsula, consistent with the negative results of soil testing. The apparent low level of environmental contamination with *T. gondii* oocysts on Phillip Island may be associated with lower cat densities. The present study attempted to compare the level of environmental contamination at sites with two different estimated cat densities, however, oocysts were not able to be detected in either case.

Figure 5.1 depicts the overall timeline of the present study and indicates when sample collection and testing occurred for each component of the research. As can be seen from the figure, much of the sample collection and subsequent testing occurred concurrently throughout the project period. Preliminary data suggesting a very high prevalence of *T. gondii* infection in feral cats was available prior to the release of the bandicoots. This information was carefully considered by stakeholders involved and it was decided that the release should go ahead as planned, as the consequent level of environmental contamination was unknown, and therefore the risk for bandicoots was still difficult to estimate. Preliminary data on the level of

*T. gondii* environmental contamination on Phillip Island (soil and feral rabbits, Chapter 3) were not available prior to the release.

Eastern barred bandicoots were released onto the Summerland Peninsula in late 2017. No bandicoots showed serological evidence of exposure to *T. gondii* prior to release. Bandicoots were trapped and tested twice post-release, and no bandicoots were found to have seroconverted to *T. gondii* over the course of the study. Limited mortality data was available for the bandicoots and only one deceased animal was recovered. This animal was not infected with *T. gondii* based on qPCR testing of tissues. Although there was no serological evidence of *T. gondii* exposure in bandicoots that were successfully trapped and tested, the possibility of bandicoots that were not trapped becoming infected and dying acutely of toxoplasmosis could not be excluded.



### 5.3 Implications for the establishment of eastern barred bandicoots at Phillip Island and future sites

Given the limitations of this study, it was not possible to clearly elucidate the risk that *T. gondii* poses to the establishment of eastern barred bandicoot populations on Phillip Island.

However, the data available from the present study suggests that the Summerland Peninsula is a suitable site for assisted colonisation of eastern barred bandicoots. No *T. gondii* seropositive bandicoots were identified throughout the course of the study, which was consistent with the absence of *T. gondii* infection in rabbits from the Summerland Peninsula. Bandicoots had excellent survivability, with approximately 50% of founders known to be alive at 16 weeks post-release; good foraging ability, as evidenced by the increase in bodyweight of most bandicoots; and reproductive success, indicated by the presence of pouch young born on the island, with at least one surviving to reproductive maturity. These early population data suggest that, on Phillip Island, although toxoplasmosis may be having a significant effect on individual bandicoots, it does not appear to be affecting them at a population level.

Toxoplasmosis was a significant cause of death in bandicoots released onto French Island in 2012, with four out of 18 bandicoots infected, including three fatalities associated with *T. gondii*. Although there were no cat density estimates available, it is thought that they were much higher than current densities on Phillip Island. There is an active and established cat control program on Phillip island, and this was ongoing throughout the study. This is likely to be the key difference between the two release sites. It is possible that eastern barred bandicoots are able to coexist with feral cats below a certain cat density, and the current state on the Summerland Peninsula may represent this situation. There is now an active cat control program on French Island and therefore toxoplasmosis risk may be lower if future assisted colonisations are attempted.

To elucidate the risk of *T. gondii* to the permanent establishment of this population of eastern barred bandicoots, further research is required. Monitoring of the population is ongoing, and this should include continued testing for *T. gondii* exposure. Any seropositive animals identified should be observed closely to gain further understanding of the effect (if any) of *T. gondii* infection on bandicoot longevity. Future studies should also focus on increasing the sensitivity of mortality surveillance, which may help to determine if *T. gondii* has a significant role in bandicoot mortality on Phillip Island. A longer duration and greater intensity of radio-tracking of individuals may help to collect more robust mortality data. However, there have been difficulties in constructing a transmitter design to allow long-term tracking with minimal side-effects in this species. This has been discussed in detail elsewhere (Coetsee et al., 2016). Alternatives, such as the use of detection dogs, could also be explored for the recovery of deceased animals. Detection dogs are increasingly used in conservation programs and have been shown to be superior to human observers alone in the recovery of animal carcasses (Mathews et al., 2013).

#### 5.4 Future directions for investigating the epidemiology of *Toxoplasma gondii* on Phillip Island

The present study provided early insights into the epidemiology of *T. gondii* on Phillip Island. Use of rabbits as a sentinel species appeared to be much more sensitive than direct detection of oocysts in soil and suggested that environmental contamination is heterogenous across the island. This may be associated with variation in cat density across the island. Unfortunately, cat density was not known at Cape Kitchen, which is the only site where *T. gondii* infected rabbits were found. Soil samples were also not collected at this site. Testing of soil from this apparently contaminated site may reveal the presence of *T. gondii* oocysts.

Environmental contamination with oocysts could also be measured by the detection of oocysts in invertebrates, such as earthworms, which may act as transport hosts (Bettioli et al., 2000b; Ruiz and Frenkel, 1980). Invertebrates, such as earthworms, may carry infective oocysts in their intestinal tract as a result of coprophagic feeding practices. This has previously been demonstrated using mouse bioassays (Ruiz and Frenkel, 1980). However, since *T. gondii* is only able to infect mammals and birds, earthworms are unlikely to become truly infected. Therefore, these species are unlikely to concentrate oocysts, and testing of invertebrates is not likely to be any more sensitive than the direct detection of oocysts in soil. For this reason, in addition to the labour associated with the collection of samples and cost of processing, surveillance of invertebrates for the presence of *T. gondii* oocysts was not attempted in this study.

Expansion of sentinel surveillance on Phillip Island, i.e. collection of more rabbits from a greater number of different sites, may help to more precisely map out the heterogeneity of *T. gondii* environmental contamination on Phillip Island. This should be paired with feral cat density estimates from each site to help to understand the relationship between cat density and the level of environmental contamination with oocysts. Furthermore, since there was a strong seasonal association with infection risk in feral cats, this relationship should also be investigated in a sentinel intermediate host (e.g. rabbits). Infection risk is expected to be strongly seasonal in rabbits. If confirmed, this may help to inform higher risk seasons for other intermediate hosts that are susceptible to toxoplasmosis.

Vertical transmission of *T. gondii* is thought to occur in rabbits, however, this occurrence has not been well characterised (Uhlíková and Hübner, 1973). Therefore, it is possible that *T. gondii* may persist in a rabbit population in the absence of environmental contamination. This

complicates the role of rabbits as a sentinel species for *T. gondii*. Vertical transmission has also been identified in other potential sentinel species, such as mice (Owen and Trees, 1998). Thus, it may be difficult to identify a sentinel species in which vertical transmission does not occur.

To the author's knowledge, there are no studies exploring the genotypes of *T. gondii* infecting feral cats in Australia. *Toxoplasma gondii* genotype is thought to be associated with virulence (Howe and Sibley, 1995). Studies have suggested that *T. gondii* genotypes are diverse in Australia, and at least in Western Australian marsupials, infection with atypical or non-archetypal type II strains is common (Pan et al., 2012; Parameswaran et al., 2010). Genotyping of the *T. gondii* strains infecting Phillip Island feral cats will help to determine the relative prevalence of different genotypes in the definitive host. This may help to determine whether the genotypes observed in native marsupials with toxoplasmosis are representative of the *T. gondii* population infecting feral cats in Australia. If the prevalence and distribution of *T. gondii* genotypes differs between the two groups, strains that are particularly virulent for marsupials may be identified.

Toxoplasmosis is a zoonosis and is therefore an important public health consideration. The high prevalence of *T. gondii* infection in feral cats on Phillip Island may constitute a risk for humans living on the island. Humans may be exposed to oocysts in soil (e.g. while gardening), or on contaminated food items (e.g. unwashed vegetables), or via ingestion of bradyzoite tissue cysts in undercooked meat (Jones et al., 2009; Weigel et al., 1999). People living on Phillip Island should ensure general principles of good hygiene (i.e. washing hands before eating/drinking, washing vegetables) are adhered to, to minimize risk. Phillip Island is an agricultural area and domestic livestock may become infected with *T. gondii* by ingesting oocysts on pasture. Future studies should determine the prevalence of *T. gondii* infection in

livestock on the island, for example via abattoir surveillance using the MAT or qPCR on tissues, to estimate potential risk for consumers. It is recommended that any meat obtained from livestock grown on the island is cooked thoroughly before consumption.

## 5.5 Concluding remarks

Overall, the present study suggests that eastern barred bandicoots may be able to establish new populations, via assisted colonisation, in the presence of feral cats. However, greater understanding is required before the findings from Phillip Island can be extrapolated to other sites. Furthermore, *T. gondii* infection is highly prevalent in feral cats on Phillip Island, and this may pose risks to vulnerable native species other than eastern barred bandicoots. Continued research into the epidemiology of this parasite on Phillip Island is indicated.

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