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Seroprevalence of *Toxoplasma gondii* in burrowing bettongs (*Bettongia lesueur*): a comparison of cat-free and cat-exposed populations

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ABSTRACT

Toxoplasma gondii is a ubiquitous protozoan transmitted by felids and infection, morbidity, and mortality occur in numerous marsupial species. This study explores the relationship between cat exposure and Toxoplasma in burrowing bettongs (*Bettongia lesueur*) in the Arid Recovery Reserve (ARR), South Australia. We estimated seroprevalence, using a modified agglutination test for *T. gondii*-specific immunoglobulins, in cat-free and cat-exposed bettong populations. Tissue samples collected opportunistically from bettong carcasses and from cats within and around the reserve were screened for *T. gondii* DNA using multiplex real-time polymerase chain reaction (M-qPCR). Two cats trapped inside the ARR tested positive (50.0%; 95% CI: 15.0–85.0%). All bettongs tested from the cat-free (n = 48) and cat-exposed (n = 19) exclosures were seronegative (95% CI: 0–7.41% and 0–16.82% respectively). We found no evidence of fatal toxoplasmosis, with all bettong carcasses negative on M-qPCR (n = 11). We propose that *T. gondii* was not detected in bettongs coexisting with cats primarily due to low exposure of bettongs at the time of sampling, possibly due to poor oocyst viability in arid conditions or low shedding by cats. Ongoing screening throughout high and low rainfall years should be conducted to better establish the risk of Toxoplasma to bettongs in the ARR.

Keywords: arid zone, Australian native marsupials, feral cats, modified agglutination test, multiplex real-time PCR, parasite, reintroduction, toxoplasmosis.

Introduction

Feral domestic cats (*Felis catus*) play a significant and ongoing role in the extinction crisis facing Australian native mammals (Woinarski *et al.* 2015). Since their introduction in 1788 (Abbott 2008), cats have thrived, with populations estimated at 2.1–6.3 million across over 99.8% of Australia (Legge *et al.* 2017). Predation is widely regarded to be the strongest direct negative effect of cats on endemic wildlife populations (Woinarski *et al.* 2015; Doherty *et al.* 2017). However, less is known about the negative and potentially exacerbating, indirect effects of this invasive species, such as disease transmission (Medina *et al.* 2014). Despite ongoing efforts, complete eradication of feral cats from the Australian mainland is considered highly unlikely in the foreseeable future (Doherty *et al.* 2017; Legge *et al.* 2017). Consequently, a more complete understanding of predator–prey interactions is crucial to both the management of feral cats and the successful conservation of threatened native species (Medina *et al.* 2014).

The zoonotic protozoan *Toxoplasma gondii* is receiving increasing interest for its potential role in population declines of native marsupials, with infection or exposure documented in numerous species (Hillman *et al.* 2016). Felids are the definitive host of this parasite, which has a worldwide distribution and can infect virtually all endothermic species (Robert-Gangneux and Dardé 2012). Several studies have found

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seroprevalence in free-ranging populations of marsupials to be positively associated with cat density (Parameswaran 2008; Hollings *et al.* 2013; Taggart *et al.* 2019).

Infection in felids is predominantly subclinical and usually follows predation of an intermediate host carrying tissue cysts (bradyzoites). Sexual reproduction of the parasite results in millions of oocysts being shed in faeces, where they sporulate to become infective to intermediate species following ingestion (Dubey 2010). Significant clinical disease has been confirmed in a range of Australian marsupials, although clinical outcome varies between species (Canfield *et al.* 1990; Innes 1997; Dubey 2010; Hillman *et al.* 2016). Outbreaks described in captive marsupials are often characterised by high rates of morbidity and mortality (Barrows 2006; Basso *et al.* 2007; Dubey and Crutchley 2008; Guthrie *et al.* 2017). Whether or not this extreme scenario extends to free-ranging populations is unclear (Hillman *et al.* 2016), with evidence of both overt disease (Obendorf and Munday 1983; Obendorf *et al.* 1996; Parameswaran *et al.* 2010; Groenewegen *et al.* 2017) and subclinical infection (Parameswaran *et al.* 2009; Pan *et al.* 2012; Fancourt *et al.* 2014; Reiss *et al.* 2015) reported.

Burrowing bettongs, *Bettongia lesueur* (Marsupialia: Potoroidae), are a medium-sized, bipedal, largely herbivorous marsupial living communally in burrows (Sander *et al.* 1997; Bice and Moseby 2008). The species has suffered drastic population declines following European settlement, with natural populations now limited to <0.01% of their former range (Short and Turner 1993). Successful reintroduced subpopulations are confined to feral predator-free islands and fenced mainland sanctuaries (Short and Turner 2000; Moseby *et al.* 2011; Richards 2012). Toxoplasmosis is included as a possible threat in the Australian Government's National Recovery Plan for burrowing bettongs (Richards 2012). Prevalence studies have so far been limited to felid-free populations, in which there was no evidence of exposure (Parameswaran 2008).

We sought to explore the relationship between exposure to felids and infection with *T. gondii* in a reintroduced population of burrowing bettongs within the Arid Recovery Reserve (ARR), South Australia (SA). We anticipated that evidence of *T. gondii* would be higher in bettongs coexisting with cats within the reserve. Through this study, we aimed to improve understanding of the indirect impacts of feral cats and the possible risk of *T. gondii* transmission involved with exposing native marsupials to cats.

Materials and methods

A modified agglutination test (MAT) was used to determine the serostatus of burrowing bettongs belonging to two populations in the ARR: one protected from cats and another coexisting with cats. To avoid overlooking individuals that might have died from acute toxoplasmosis, bettong carcasses collected from within the reserve were tested for *T. gondii* DNA using a multiplex real-time polymerase chain reaction (M-qPCR). Tissue samples collected from cats culled as part of a local feral predator control program and incursions into the reserve were also screened for *T. gondii* DNA.

Study site

Our study was conducted in the ARR, an ecosystem restoration programme in SA (30°29′S, 136°53′E), approximately 20 km north of the Roxby Downs township. The 123 km^2 reserve is surrounded by wire-mesh fencing effective at preventing entry of cats and foxes (*Vulpes vulpes*). Predator-proof fencing is used to section the area into several exclosures containing different native and non-native species (Moseby and Read 2006). Those relevant to this study include the Cat-Free Paddock (CFP), where bettongs reside without cats, and two Cat-Exposed Paddocks (CEP 1 and CEP 2), in which the two species coexist (Fig. 1). Mean annual rainfall is 138.0 mm, although rainfall is erratic (Bureau of Meteorology 2020*b*). Exclosures are within the same land system with minimal variation in climate between sites.

Bettongs ($n = 30$) were translocated to the CFP (14 km^2) in 1999–2000 from two felid-free islands (Moseby *et al.* 2011). Regular active monitoring for predator incursions has since been performed (Moseby and Read 2006; Moseby *et al.* 2019). This acted as the source population for translocations to CEP 1 (26 km^2) in 2014 (n = 352) (Moseby *et al.* 2019), where a population of feral cats

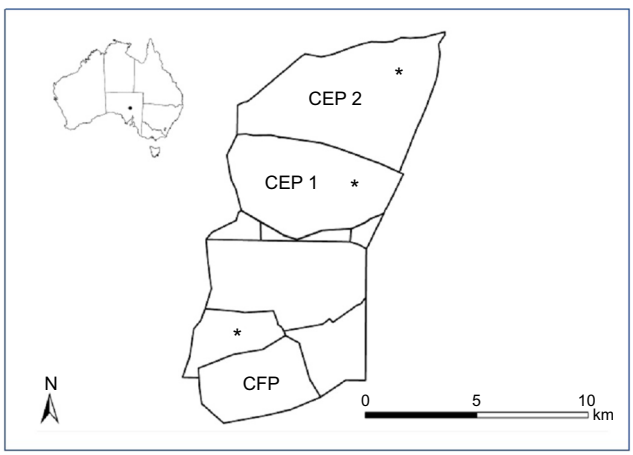

Fig. 1. Schematic of the Arid Recovery Reserve in South Australia highlighting the exclosures from which bettongs and cats were tested in our study. The solid lines denote predator-proof fencing. CFP, Cat-Free Paddock; CEP 1, Cat-Exposed Paddock 1; CEP 2, Cat-Exposed Paddock 2. The locations of cats tested within the ARR are indicated by asterisks.

(0.08–1.92 cats km^{-2}) has been maintained as part of research into the potential for alien predators and threatened native prey species to coexist (Moseby *et al.* 2019). This is achieved through the addition of cats from the surrounding area, incursions, breeding, and culling (Moseby *et al.* 2019; K. Moseby, pers. obs.). At the time of this study, there were an estimated 71 bettongs in the CFP, and 43 bettongs and three cats (0.16 cats km^{-2}) in CEP 1. In CEP 2 (37 km^2), 10 feral cats were known to be present in 2008/2009 (0.27 cats km^{-2}) (Schroeder *et al.* 2015) alongside bettongs, and camera traps and spoor counts suggest that cats have been present continuously since that time (K. Moseby, pers. obs.).

Sample collection

Bettongs were captured in treadle-operated Sheffield cage traps baited with rolled oats and peanut butter and set along road networks in the CFP (n = 83) and CEP 1 (n = 141) for four consecutive nights in May 2019. Individuals were observed in the trap before being transferred into a handling bag. They were assessed for clinical signs of toxoplasmosis, including obvious neurological (e.g. head tilt), respiratory (e.g. dyspnoea), and ocular abnormalities (e.g. blindness), and marked lethargy. Up to 0.5 mL of blood was collected from the lateral coccygeal vein using a 23-gauge 19 mm winged infusion set into a serum tube and stored on ice (Vogelnest and Portas 2008). Individuals were conscious and kept in the handling bag during venepuncture with their heads covered to minimise stress, with the procedure lasting less than 2 min per bettong. Observations and sampling were performed by the same two veterinarians.

Between November 2017 and April 2019, bettong carcasses found within CFP and CEP 2 were collected and stored at −20°C. We selected carcasses that were sufficiently intact for tissue sampling, rejecting extremely decomposed specimens to reduce the likelihood of false negative results associated with expected DNA degradation. Carcasses were thawed prior to necropsy and examined for gross pathology suggestive of toxoplasmosis, including pulmonary congestion, consolidation or oedema, splenomegaly, hepatomegaly, patterning to the liver parenchyma and erythema or haemorrhages to abdominal or thoracic organs (Canfield *et al.* 1990; Bettiol *et al.* 2000) A total of 5 g of tissue was sampled from the tongue, skeletal muscle (biceps femoris and/or quadriceps), brain, heart, lung, and eyes depending on availability from each individual, and refrozen.

Tissue samples were obtained opportunistically from cats culled as part of a feral predator control programme within the ARR and in the surrounding area, including the Roxby Downs township. In August and September 2019, two cats were trapped within CEP 2 and a third as an incursion into an exclosure separate from the bettong populations included in this study (Fig. 1). A combination of liver, tongue, heart, and skeletal muscle (quadriceps) samples totalling 5 g were collected shortly after euthanasia and immersed in 70% ethanol. In addition, liver samples from six cats previously collected and stored in ethanol in 2018 were also tested as part of our study. One of these cats inhabited CEP 1 and the remainder had been caught outside of the reserve.

Modified agglutination test (MAT)

Within 6 h of sampling, whole blood samples were centrifuged, and serum was transferred to microcentrifuge tubes for storage at −20°C. Serological testing took place at the Faculty of Veterinary and Agricultural Sciences, University of Melbourne. A commercially available MAT (Toxo-Screen DA, bioMérieux, Marcy-l'Etoile, France) was used to analyse serum for the presence of *T. gondii*-specific immunoglobulin G (IgG), following a modified protocol used by the Animal Health Laboratories of the Department of Primary Industries, Parks, Water and Environment (DPIPWE), Tasmania, Australia. Serial four-fold dilutions of the samples were obtained by mixing phosphate buffered saline with 25 µL of each serum sample, resulting in final dilutions of 1:16, 1:64 and 1:256. Positive and negative controls supplied with the kit were used at 1:16 dilution. Mercaptoethanol (0.2 mol L^{-1}) was added to denature IgM and suppress non-specific agglutination in all sample wells. Formalin-treated *Toxoplasma* antigen was added, which agglutinates in the presence of *T. gondii*-specific IgG.

There is no established serological cut-off for this test in bettongs and so a positive reaction was concluded if a pink mat of agglutinated antigen covered more than half of the bottom of the sample well at a dilution of at least 1:64. This follows the protocol used by Mt. Pleasant Laboratories (a NATA accredited facility) and is consistent with published studies using this test in Australian marsupials (Obendorf *et al.* 1996; Miller *et al.* 2000; Hartley and English 2005; Hollings *et al.* 2013; Fancourt *et al.* 2014). Positive and borderline reactions were repeated on a second serum sample and diluted further to 1:1024 and 1:4096.

Sensitivity and specificity values have not been calculated for the MAT in bettongs. Extrapolating from available data, we estimate a specificity of 93% in line with that determined by Hillman *et al.* (2017) for other marsupial species. Sensitivity could not be calculated in this study, and therefore we estimate 96% based on evaluations in domestic cats (Adriaanse *et al.* 2020).

Multiplex real-time polymerase chain reaction (M-qPCR)

Tissue digestion and DNA extraction using the ISOLATE II Genomic DNA Kit (Bioline) were carried out on bettong and feline tissue samples as described by Adriaanse *et al.* (2020).

From each individual, 5 g of pooled tissue sample was used for DNA extraction. DNA concentration was increased by performing the elution step twice (50 μL each) to a final elution volume of 100 μL. Genomic DNA were subjected to M-qPCR in duplicates on a MIC Personal qPCR Cycler (Bio Molecular Systems, Upper Coomera, Queensland, Australia) as described by Adriaanse *et al.* (2020). Genomic DNA from *T. gondii* tachyzoites (Walter and Elizabeth Hall Institute, Melbourne, Australia) was used as positive and non-template controls for each reaction. Probes and primers used are outlined in Table 1. A Ct threshold of <35 was needed to consider a sample positive. For analysis, results were converted to a binary variable (positive or negative).

Data analysis

All statistical analyses were performed using the epidemiological calculator 'Epitools' (Sergeant 2018) and the 'Winpepi' software suit (Abramson 2011).

Ethical considerations

Approval was obtained from the SA Wildlife Ethics Committee (project 2/2019) and the Animal Welfare and Ethical Review Board, University of Edinburgh. Import (14749548) and wildlife research permits were obtained for Victoria (DELWP (No.10009104). Field work and export of samples were carried out under Arid Recovery's research permit (U26497-6).

Results

Bettongs

Bettongs were trapped in 44.6% (37/83) of traps set in the CFP and 9.2% (13/141) in CEP 1. An estimated 67.6% of all bettongs in the CFP (48/71) and 44.2% of bettongs in CEP 1 (19/43) were tested using the MAT. There was no statistically significant difference between the proportion of males (CFP: $n = 13$; CEP 1: $n = 9$) and females (CFP: $n = 35$; CEP 1: $n = 10$) (Upton's modified Chi-square test, $\chi^2 = 2.502$; 1 d.f.; $P = 0.114$) or the proportion of subadults (<18 months) (CFP: $n = 1$; CEP 1: $n = 1$) and adults (>18 months) (CFP: $n = 47$; CEP 1: $n = 18$) (two-tailed Fisher's exact test; $P = 0.490$) when comparing the sample cohort from each exclosure (Campbell 2007).

The opportunity for assessment of bettongs in cage traps and in the hand was limited, but no obvious clinical signs associated with toxoplasmosis were observed. All serum samples were negative for *T. gondii*-specific IgG based on a cut-off dilution of 1:64. An apparent seroprevalence of 0% ($n = 48$; 95% CI: 0–7.41%) was calculated for the cat-free population (CFP) and 0% ($n = 19$; 95% CI: 0–16.82%) for the cat-exposed population (CEP 1) using a Wilson score interval (Brown *et al.* 2001).

Ten adult bettong carcasses from CEP 2 and one from the CFP were suitable for necropsy and sampling. No gross pathology associated with toxoplasmosis was observed and all tissue samples were negative for *T. gondii* DNA by M-qPCR.

Feral cats

Of the four cats caught within the ARR, two tested positive for *T. gondii* DNA by M-qPCR (50.0% of cats trapped at ARR; 95% CI: 15.0–85.0%): one in CEP 2 and one as an incursion in another exclosure. Both were trapped in September 2019 and a full set of tissue samples were collected. No cat incursions occurred in the CFP where bettongs were sampled. Two of the five liver samples collected from cats trapped outside the ARR in 2018 also tested positive (40.0% of cats trapped outside of ARR; 95% CI: 11.8–76.9%). Both resided within the Roxby Downs township.

Table 1. Targets, primers, probes and concentrations used in a multiplex qPCR to screen burrowing bettongs (*Bettongia lesueur*) and feral cats (*Felis catus*) for infection with *Toxoplasma gondii*.

Target		Oligonucleotide sequence 5′-3′	Gene target	F.C. (nM)	Source
Toxoplasma gondii	Forward	AGAGACACCGGAATGCGATCT	529 repeat element	200	Adriaanse *et al.* (2020)
	Backward	CCCTCTTCTCCACTCTTCAATTCT		100	
	Probe	/5Cy5/ACGCTTTCC/TAO/TCGTGGTGATGGCG/3IAbRQSp/		100	
Equine Herpes Virus 4	Forward	GATGACACTAGCGACTTCGA	gB	80	Llewellyn *et al.* (2016)
	Backward	CAGGGCAGAAACCATAGACA		80	
	Probe	/56-ROXN/TTTCGCGTGCCTCCTCCAG/3IAbRQSp/		200	
Felis catus	Forward	CGACCTCGATGTTGGATCAG	Large subunit rDNA	100	Traub lab (2019), unpubl. data
	Backward	GAACTCAGATCACGTAGGACTTT		100	
	Probe	/56-FAM/CCCGATGGT/ZEN/GCAGCAGCTATCAA/3IABkFQ/		200	

Discussion

The objective of our study was to explore the relationship between exposure to feral cats and detection of *T. gondii* in burrowing bettongs in the ARR. To the authors' knowledge, these findings represent the first seroprevalence study of *T. gondii* in a population of burrowing bettongs coexisting with cats. All bettongs tested from both cat-exposed and cat-free populations were negative for *T. gondii* infection or exposure using the MAT and M-qPCR. However, we did detect evidence of infection in the local feral cat population.

We detected *T. gondii* in the feline population both inside the ARR and in the Roxby Downs township (50.0% of cats trapped at ARR (95% CI: 15.0–85.0%) and 40.0% of cats trapped outside of ARR (95% CI: 11.8–76.9%)). Unfortunately, our small sample sizes hindered precise estimation of prevalence in these groups. Comparisons with other studies using PCR to determine prevalence in Australian feral cats are problematic due to both our limited dataset and the significant geographical variation observed. Dickson (2018) estimated 24.4% prevalence across the continent (n = 234) but identified just 6.3% in Roxby Downs (n = 16). Adriaanse *et al.* (2020) detected *T. gondii* DNA in 79.5% of cats on Phillip Island, Victoria (n = 161), whereas Adams (2003) recorded a prevalence of just 4.9% in Western Australia (WA) (n = 268). Despite this high variability and the challenges previously highlighted, our sampling confirms the presence of *T. gondii* in the definitive feline host at our study site. However, although cats have been present continuously in CEP 2, we cannot be sure of temporal overlap between the positive cat trapped in September 2019 and bettong carcasses collected between November 2017 and April 2019, and therefore cannot confirm that exposure occurred.

Prevalence in the feline population outside the ARR may have been underestimated as liver had been exclusively sampled from these cats. Bradyzoites have lower tropism for liver in felids, leading to the potential for false negatives (Dubey 1997). This could explain the lower seroprevalence detected in Roxy Downs by Dickson (2018), as, similarly, only liver was tested. In three of the cats captured inside the ARR, we were able to sample a range of tissues from optimal bradyzoite predilection sites, which were pooled for testing, thus increasing the likelihood of detection. It is therefore possible that the higher prevalence found in the ARR is a more accurate reflection of feline prevalence in this region, although again our relatively small sample size should be noted.

Several studies in Australia have shown an increased seroprevalence in free-ranging marsupial populations in areas with higher densities of cats (Parameswaran 2008; Hollings *et al.* 2013; Taggart *et al.* 2019; Taggart *et al.* 2021). Although cat numbers were relatively low at the time of sampling (0.16 cats km^{-2}), most of the bettongs sampled in this exclosure (18/19) were known from previous trapping data to have been alive when density peaked at 1.92 cats km^{-2} in January 2018 (K. Moseby, unpubl. data). This is much higher than the mean density of feral cats inhabiting natural environments of Australia (mainland and islands), estimated at 0.27 cats km^{-2} (95% CI: 0.18–0.45 cats km^{-2}) (Legge *et al.* 2017). However, we found no difference in seroprevalence between the cat-free and cat-exposed bettong populations, estimating 0% in both the CFP and CEP 1.

Our estimates are consistent with 0% seroprevalence of *T. gondii* reported by Parameswaran (2008) in two populations of burrowing bettongs, although notably these were from Faure Island (0/28) and Barrow Island (0/14), both felid-free islands in WA. Although seropositive brush-tailed bettongs have been identified in two populations in WA (5.8% n = 153; 1.4% n = 73) (Parameswaran 2008), surveys of other bettong species in Tasmania (Portas *et al.* 2014) and various locations in WA (Parameswaran 2008; Skogvold *et al.* 2017) have failed to detect evidence of *T. gondii* infection. Given these reports of very low seroprevalence in bettongs, it is possible that our sample sizes in the CFP and CEP 1 were not large enough to detect a positive individual, despite the high percentage of both bettong populations included in the survey (67.6% and 44.2% respectively).

In CEP 1, bettongs were captured in only 9.2% of set traps, all of which geographically clustered within one half of the exclosure. This might indicate a distribution preference by bettongs to avoid habitat occupied by cats, which could also have affected their exposure to oocysts. However, monitoring of feline movements in the exclosure has detected cats on every kilometre of track transects and on over 50% of camera traps (Moseby *et al.* 2019), making this scenario unlikely.

The level of environmental contamination with oocysts is dependent not only on feline abundance and prevalence, but also oocyst shedding. Although the detection of *T. gondii* DNA confirms the presence of infection, it does not indicate whether that individual is shedding. A single cat can excrete millions of oocysts (Dubey 2001); however, shedding typically occurs for only 1–2 weeks following initial infection and is affected by numerous host and parasite factors (Dubey *et al.* 1977; Dubey 1995). Consequently, it is estimated that, at any given time, less than 1% of cats are expected to be excreting oocysts (Dubey and Jones 2008). Therefore, there is only a very small probability of there being a cat shedding in the ARR at the time of sampling and so the risk of bettong exposure to *T. gondii* is low.

A key factor in the transmission of *T. gondii* from cats to intermediate hosts is sporulation and survival of oocysts in the environment. Although sporulated oocysts are environmentally robust, prolonged viability occurs in cool, moist soil or water (Yilmaz and Hopkins 1972; Frenkel

et al. 1975). Oocyst survival is proposed to account for patterns in *T. gondii* infection worldwide, with higher prevalence observed in tropical or temperate regions (Meerburg and Kijlstra 2009; Montazeri *et al.* 2020). Numerous studies correlate seropositivity in intermediate and definitive hosts with rainfall (Almería *et al.* 2004; Gamarra *et al.* 2008; Afonso *et al.* 2013). In Australia, lower seroprevalence is recorded in cats inhabiting semiarid compared to temperate parts of Victoria (Coman *et al.* 1981) and Tasmania's cooler, wetter climate is proposed to explain a generally higher detection of *T. gondii* in resident marsupial species and cats (Fancourt *et al.* 2014; Reiss *et al.* 2015). This is supported by climate and microclimate models demonstrating low annual rainfall and high air and soil temperatures to be the most significant factors restricting distribution of this parasite in Australian cats (Dickson 2018).

Laboratory experiments confirm a significant reduction in survival and infectivity of oocysts at high temperatures (Dubey 1998). Non-sporulated oocysts are particularly fragile, with exposure to 37°C proving lethal after 24 h (Dubey *et al.* 1970). Summer temperatures in the ARR regularly exceed this and therefore oocysts may only be present and infective during cooler winter months (Bureau of Meteorology 2020*a*). Oocysts are also susceptible to desiccation in low humidity. Lélu *et al.* (2012) found only 7.4% of oocysts surviving after 100 days in dry soil, corresponding to 281 mm of rain per year. Average yearly rainfall for the Roxby Downs area is just 138.0 mm and the region has recently suffered several years of drought, recording the lowest annual rainfall in nearly two decades in 2019 (Bureau of Meteorology 2020*b*). Such conditions are likely to have further diminished the number and infectivity of oocysts present in the environment, reducing the likelihood of transmission from cats to bettongs at the time of our study.

As well as modulating oocyst survival within the environment, climatic variations can influence transmission by acting on host population composition and interactions (Afonso *et al.* 2013). For example, expansion of both small mammal and feline populations occurs following increased rainfall (Stenseth *et al.* 2002; Legge *et al.* 2017). Such conditions can amplify the parasite in both populations through increased predator–prey interactions (Lélu *et al.* 2010), vertical transmission (Marshall *et al.* 2004) and increased environmental burden as young cats are infected and shedding for the first time (Dubey *et al.* 1977; Dabritz *et al.* 2007). This scenario was proposed to have occurred in the Gibson Desert Nature Reserve, WA, where prevalence in cats increased from 0% in 2000 to 66.7% in 2001 following an unusually high winter rainfall (Adams 2003). Studies have detected a seasonality in the risk of infection in cats (Adriaanse *et al.* 2020), the proportion of cats in a population shedding (Herrmann *et al.* 2010; Schares *et al.* 2016), and the detection of oocysts in soil (Wit *et al.* 2020). In Roxby Downs, seasonal fluctuations in feral cat density have been recorded and linked to peaks and troughs in rabbit numbers in the area (Read and Bowen 2001). Therefore, repeat sampling during cooler, wetter periods in the ARR might reveal different results in both the feline and bettong populations.

Seroprevalence estimates in bettongs were based on results of a MAT, which exclusively agglutinates IgG. IgG is generally not detectable in sera until 1–3 weeks after initial infection (Johnson *et al.* 1989; Bettiol *et al.* 2000; Robert-Gangneux and Dardé 2012). Therefore, sensitivity during the acute phase of infection is very low (Johnson *et al.* 1989; Lynch *et al.* 1993; Bettiol *et al.* 2000). If *T. gondii* was present in the bettong population and causing acute toxoplasmosis with high rates of mortality, infected individuals may have died before detectable levels of IgG were present in their sera, leading to a falsely low seroprevalence. To address this limitation, we screened opportunistically collected deceased bettongs using M-qPCR. We did not detect *T. gondii* DNA in any of the individuals sampled ($n = 11$), including 10 that had coexisted with cats. Several studies have used PCR to detect *T. gondii* in marsupials (Parameswaran *et al.* 2010; Pan *et al.* 2012; Hillman *et al.* 2017). To minimise false negatives, we included relatively large quantities (5 g) of pooled tissue samples from several sites commonly infiltrated by bradyzoites (Dubey 1997). We also selected carcasses that were not significantly decomposed and performed necropsies straight after defrosting to minimise DNA degradation. Therefore, it is highly likely that these bettongs were free from infection at the time of death. However, given the small sample size and the expectation that a proportion of dead bettongs are not detected, the possibility that bettongs are not succumbing to fatal acute disease cannot be excluded.

It is also possible that seropositive bettongs were not detected in our seroprevalence survey because they are more frequently preyed upon by cats. Latent toxoplasmosis in intermediate hosts has been correlated with adaptive manipulation, the parasite-induced alteration of host behaviour to specifically benefit the parasite (Poulin 2010). In latently infected rats and mice, this can manifest as both a loss of aversion to cat odours and a simultaneous attraction to them (Berdoy *et al.* 2000; Vyas *et al.* 2007). Although further research is needed, there is a growing body of literature suggesting that a similar scenario may occur in other species in the wild (Kreuder *et al.* 2003; Poirotte *et al.* 2016).

Conclusions and recommendations

The detection of *T. gondii* in cats in the region of the ARR is concerning and highlights a potential disease threat to valuable reintroduced populations of native species. We

propose that the apparent absence of *T. gondii* exposure in bettongs coexisting with cats may have arisen due to a low risk of bettong exposure to this parasite at the time of sampling. This may be due to poor oocyst viability in arid conditions exacerbated by recent drought, and the low probability of oocyst shedding by cats inhabiting the exclosure. Screening of soil samples for the quantity and infectivity of oocysts using qPCR would be beneficial to further explore this theory (Lélu *et al.* 2012). Although we found no evidence of bettongs dying from acute toxoplasmosis, this scenario cannot be excluded, and we recommend further screening of carcasses for *T. gondii* DNA. Crucially, we recommend that ongoing testing be performed through a range of seasonal conditions and during drought and high rainfall years. Ideally, this would be extended to include other susceptible species inhabiting the reserve. These efforts would account for the effects of climate on the epidemiology of *T. gondii* and establish the risk of transmission of this important parasite to reintroduced populations of vulnerable native species.

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Data availability. The data that support this study will be shared upon reasonable request to the corresponding author.

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